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ON THE SO-CALLED NEISSER-WECHSBERG INHIBITING PHENOMENON IN BACTERICIDAL IMMUNE SERA

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A. HISTORICAL

In the year 1901 Neisser and Wechsberg described a peculiar function of immune sera, which they called "Complementablenkung," and which later has been named the "Phenomenon of Neisser and Wechsberg." The phenomenon consisted in the observation that, whereas small or medium doses of bactericidal sera exhibited the bactericidal function with the homologous strain, the larger doses were without effect. Thus an inactivated immune serum against Vibrio Metschnikoff with added complement, showed bactericidal action in doses of 0.05 to 0.0025 cc., but in larger doses it had no such effect. Likewise, Neisser and Wechsberg showed that the dose of complement-bearing serum, which was sufficient to activate a certain dose of an inactivated serum, was not capable of doing so when larger doses of immune serum were added.

The explanation of this paradoxical serum function was sought, by the authors, in the great richness of the immune serum in bactericidal amboceptors. These antibodies, which, in the immune serum, must be present in far greater quantities than the complement in the activating serum, were supposed to unite with the complement and form thereby a lysin that dissolves the bacillus after becoming attached to it; but on account of the predominance in the number of the amboceptors, there being insufficient complement to satisfy all of the amboceptors, some of the latter were assumed to remain as unaltered amboceptors, free of complement, and thus devoid of bactericidal function.

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When under these conditions, fixation between the serum bodies and the bacilli takes place, and if we assume that the bacilli have no greater tendency to attach themselves to the lysin than to the free amboceptor, the possibility may be considered that some of the bacilli will combine with the lysin and will be dissolved, while others will unite with the free amboceptors and will not be affected. The latter bacilli will survive, and the test will not show bactericidal function. This result would be the more likely under the assumption that effective lysin has a lesser avidity for the bacilli than the free amboceptor. If this supposition is true, the greater number of bacilli will be attached to the ineffective, free amboceptor, while the effective lysins will remain unused, and these will have no opportunity of bringing about the bactericidal effect. When, on the other hand, the amount of amboceptors is small, as in a diluted serum, all of the amboceptors will be supplied with complement. Consequently, there will be only effective lysin in the test and the bactericidal effect will occur.

Thus, according to Neisser and Wechsberg, the surplus of amboceptors in an immune serum brings about the inhibition of the bactericidal action, because this surplus makes the effective utilization of complement impossible ("Complementablenkung").

Contrary to Neisser and Wechsberg, Gruber supposes that the immunization calls forth antibodies that act antibactericidally and antihemolytically. He, therefore, denies the significance of the surplus amboceptors.

Lipstein, however, entertains the same opinion of the phenomenon as Neisser and Wechsberg, and shows that the phenomenon is of a strictly specific character, that it is not called forth by normal serum-bodies, and that the agglutination does not play any part in the origin of the phenomenon.

By absorbing the bactericidal serum with the homologous bacillus, Lipstein succeeded in robbing the serum of its inhibiting action. He therefore supposes that the serum bodies that bring the phenomenon about, are of the nature of an amboceptor, and he simply identifies them with the bactericidal amboceptors.

Levaditi rejects the theory of the lysin, showing that serum, wherein the homologous bacteria have been sensitized, loses all bactericidal effect, although, according to the theory of Neisser and Wechsberg, such serum ought to contain numerous unattached lysins, after the bacteria are removed in the centrifuge. To explain the phenomenon Levaditi supposes that ineffective amboceptors ("ambocepteur inactive") are produced in the course of the immunization. Furthermore, he supposes that these amboceptors have a greater tendency to attach themselves to the bacilli, than the effective ones.

Gay believes that precipitating antibodies arise during the immunization and that these antibodies, with the homologous antigen, form a precipitate, that absorbs the complement. He further shows that the phenomenon of Neisser and Wechsberg also can be found in hemolysis tests.

Sormani likewise observed the phenomenon in hemolysis tests, when very strong solutions of serum are used for the sensitizing of the red blood corpuscles, and furthermore, he found that the blood corpuscles, under these circumstances, could be dissolved mechanically by shaking, without the influence of complement. He calls this phenomenon specific fragility ("specifische Sproedigkeit").

Microscopically Sormani could show that the corpuscles, after the treatment in serum, were shrunken and jagged. He therefore surmises that something happens to the surface of the corpuscles during the treatment and that this "something" tends to make the corpuscles more resistant to the influence of the hemolytic antibodies. He supposes that the cause is to be found in a precipitation of the albumin of the surface and that this precipitation forms a covering around the corpuscle. Through this supposition Sormàni also explained for himself the specific fragility since it was reasonable enough to assume that the hardened corpuscles could be broken by vigorous shaking. Likewise, it was a natural assumption that the hemolytic antibodies could be hindered in their action, when the corpuscle was protected by a more or less thick covering of coagulated albumin.

The theory of Sormani, however, has a drawback in a fact that cannot be easily explained. Sormani himself stated that corpuscles that had been treated with concentrated serum, showed neither the phenomenon of Neisser-Wechsberg nor that of specific fragility. He tried to explain this fact through the theory that the covering of albumin in this case is so thick, that it cannot be broken mechanically, and that the surface gets so shrunken that small pores are formed, through which complement forces itself into the corpuscles and causes hemolysis.

That the precipitating faculty of the serum is of importance for its inhibiting action, Sormani shows in his statement that the phenomenon of specific fragility and that of Neisser and Wechsberg, stand in direct relation to the precipitating power of the serum. Contrary to Gay, however, he does not believe that the specific precipitates absorb complement. Thus, even large quantities of a specific precipitate (sheep serum in rabbit immune serum) added to his hemolytic mixtures did not diminish the force of his complements. Only if a mixture of the emulsified precipitate and complement was kept in the incubator for one hour, was the complement inactivated, but not if the mixture remained for the same time in the room. Consequently the absorption of complement by the precipitate was not strong enough to explain the lacking hemolysis.

The phenomenon of the inhibition of the bactericidal action has also been studied earlier in this institute. Brekke studied the occurrence of the phenomenon in sera from typhoid patients. His results are somewhat contrary to those of Neisser and Wechsberg, as Brekke finds the phenomenon so seldom (in 22.2 per cent of the sera) that he does not consider it a specific phenomenon. He also calls attention to the fact that it is not always possible, as was supposed by Neisser and Wechsberg, to suppress the bactericidal function of an active immune serum by adding more immune serum to the test, and that a large dose of a strong immune serum does not always inhibit the action of complement.

Brekke, furthermore, showed that there was no direct relation between the bactericidal and the inhibiting titers of his sera. The inhibition of the bactericidal function was found in weak bactericidal sera as well as in strong ones. In several sera the inhibiting function could not be found, although they showed very high bactericidal titers, such as 0.0000001.

Since he was dealing with sera, which undoubtedly contained a considerable amount of bactericidal antibodies, Brekke had reason to claim that all of these sera should exhibit the phenomenon of Neisser and Wechsberg, if the theory of these authors was correct. As this was not the case, Brekke rejected that theory and sought another explanation. This he thought to find in the theory of the complementoids. These bodies are considered to be partly destroyed complements that possess the haptophore group unaltered, while their zymophore group is destroyed. They can unite with the amboceptor like a complement, but they cannot act as one. Consequently, the bacilli attached to an amboceptor-complementoid will not be dissolved but will remain alive. Now Brekke never saw the phenomenon in active sera, but only in such that had been inactivated through heating. He therefore supposed that the inhibiting phenomenon was due to the complementoids that had been transformed from the complements during the heating. Consequently, Brekke had to consider the phenomenon as an un-specific one throughout.

B. OWN INVESTIGATION

1. Introduction

During the work on the classification of dysentery-bacilli cultivated in Bergen and vicinity, the bactericidal test tube reaction was used as a method of separating the bacillary groups. It then turned out that the dysentery bacilli were well suited for the bactericidal test and good objects for the study of the phenomenon of Neisser and Wechsberg. This phenomenon occurred so frequently in the sera employed, that it must be considered as a regular faculty of the dysentery-immune sera from animals. It was, therefore, natural to put together the results of these tests with a special bearing upon the inhibition of the bactericidal action and to try to find out fixed rules for the appearance of the phenomenon, and perhaps bring forth facts toward its explanation.

Further special experiments were carried out in order to ascertain the dependence of the phenomenon on amboceptors and complements, its importance for the total bactericidal action of an immune serum and its variation during the immunization. Finally, the question was taken up whether the phenomenon is due to already known antibodies, or is brought about by unknown ones.

2. Technic

The sera employed were the inactivated sera of rabbits that had been given repeated intravenous injections of bacillary emulsions in normal saline solutions. The injections were begun with ⅕ to ½ eose of an agar slant culture and this amount was increased up to several whole cultures. The complement-bearing serum was obtained usually from guinea-pigs; in some of the tests, however, fresh human sera have been used on account of the scarcity of animals.

Before the reaction itself, a preliminary test was always carried out to determine the bactericidal action of the normal serum as well as its power of activating the immune serum.

An example of the preliminary test of the normal, complement-bearing serum is presented in table 1.

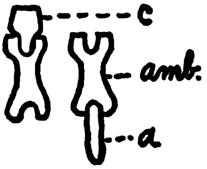

Fig 1.

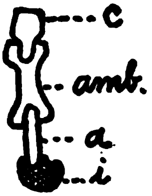

Fig 2

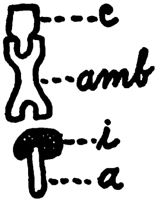

Fig 3

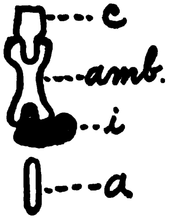

Fig 4

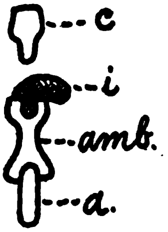

Fig 5

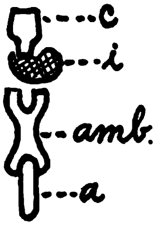

Fig. 6

C = Complement
amb. = bactericidal amboceptor
a = antigen
i = inhibiting antibody

In the first six tubes of the series are mixed broth, the unit dose of bacteria (1/8000 of an oese) and the normal serum in quantity diminishing by the usual geometrical progression. The

TABLE 1

Protocol of a preliminary test of the direct bactericidal power and of the "activating" power of normal complement-bearing serum

TUBE	AMBOCEPTOR	COMPLEMENT	ANTIGEN	BROTH	COLONIES
	cc.	*cc.*	*oese*	*drops*	
1	0	0.05	1/8000	2	0
2	0	00.025	1/8000	2	0
3	0	0.0125	1/8000	2	0
4	0	0.0063	1/8000	2	∞
5	0	0.0032	1/8000	2	∞
6	0	0.0016	1/8000	2	∞
7	0.001	0.05	1/8000	2	0
8	0.001	0.025	1/8000	2	0
9	0.001	0.0125	1/8000	2	0
10	0.001	0.0063	1/8000	2	0
11	0.001	0.0032	1/8000	2	About 1000
12	0.001	0.0016	1/8000	2	∞
13 control	0	0	1/8000	2	∞

TABLE 2

Protocol of the titration of a bactericidal immune serum (ambocepter)

TUBE	AMBOCEPTOR	COMPLEMENT	ANTIGEN	BROTH	COLONIES
	cc.	*cc.*	*oese*	*drops*	
1	0.1	0.005	1/8000	2	∞
2	0.05	0.005	1/8000	2	∞
3	0.025	0.005	1/8000	2	∞
4	0.0125	0.005	1/8000	2	∞
5	0.0063	0.005	1/8000	2	About 100
6	0.0032	0.005	1/8000	2	0
7	0.0016	0.005	1/8000	2	0
8	0.0008	0.005	1/8000	2	0
9	0.0004	0.005	1/8000	2	0
10	0.0002	0.005	1/8000	2	0
11	0.0001	0.005	1/8000	2	0
12	0.00005	0.005	1/8000	2	0
13	0.000025	0.005	1/8000	2	0
14	0.0000125	0.005	1/8000	2	About 100
15	0.0000063	0.005	1/8000	0	Many 1000
16	0.0000032	0.005	1/8000	2	∞
17	0.0000016	0.005	1/8000	2	∞
18	0.0000008	0.005	1/8000	2	∞
19	0.0000004	0.005	1/8000	2	∞
20	Complement control	0.005	1/8000	2	∞
21	Saline control	0.005	1/8000	2	∞

mixture in the tubes 7 to 12 duplicate those of the first six, excepting that an effective quantity of the bactericidal immune serum has been added to each mixture. The bactericidal titer of the normal serum ("complement") is seen to be 0.0125 (tube 3), and this represents the total bactericidal titer of this serum. Its activating titer lies between 0.0063 and 0.0032.

In table 2 is presented the protocol of the main test of the bactericidal power of the immune serum, in which the same reagents that were employed in the preliminary test were used.

The bactericidal titer of this serum is seen to be 0.0000125, the titer of inhibition is 0.0063.

This is the technic that was employed where the bactericidal action of a serum against a certain bacillus was tested. If it is desired to determine only the minimal inhibiting dose of the immune serum, it is sufficient to use an amount of complement that we know to be large enough to activate the immune serum. In this case it does not matter if this dose is so large that it has a bactericidal action of its own, because, even so, the degree of inhibition can be determined. In the following tests advantage was now and then taken of this fact.

3. Specificity of the inhibition

Numerous tests have been carried out according to the described method, and the results of some of them are shown in table 3. The bacteria used were:

- *B. dysenteriae*
  - Group I. Bacillus of Shiga
  - Group II.
    - Bacillus Flexner and Strong,
    - and bacillus Y.
  - Group III.
- *B. typhosus*
- *Vibrio cholerae*
- *B. metacoli* (Bacillus no. 1, of Morgan)

These strains of bacteria have been tested in their homologous sera and for the greater part, also, in heterologous sera. Furthermore, all, with the exception of the last two, have been tested in normal rabbit sera.

The results of these tests may be stated as follows: Normal serum shows a considerable bactericidal action against the dysentery bacilli of group I and II, but no inhibiting action can be discovered in the doses smaller than 0.1 cc. Against dysentery bacilli of group III normal serum has no bactericidal action. Consequently, the question as to the existence, here, of the function of inhibition must be left open. Against typhoid bacilli, normal serum shows some bactericidal power, but no inhibiting action.

The serum against dysentery bacilli of group I (Shiga) has a considerable inhibiting action and a strong bactericidal power against the homologous organism. Against the strains of group II, it has some bactericidal power and with some of these strains it exhibits the phenomenon of inhibition.

Against dysentery III and typhoid bacilli no bactericidal action whatever is seen in the serum.

The serum against dysentery bacilli of group II has no inhibiting action against the Shiga strain. It will be seen that the sera showing the best bactericidal action also show the best inhibition.

Against the strains from group II the group II sera show a different action. The highest titers of inhibition are always to be found in tests between a serum and its homologous strain, or a closely related strain.

As group II is composed of strains that show some individual differences, it is only natural that there should be some variance in the results of the inhibiting reaction in this group. Neither of the sera of group II shows any bactericidal action against the strains of group III, nor typhoid bacilli.

The group III serum had the same bactericidal action, without inhibition against the strains of groups I and II as had a normal serum, but it showed a strong inhibiting and bactericidal action against the homologous strain from this group.

A typhoid serum showed a normal bactericidal effect without inhibition against the strains of groups I and II, and no effect against the group III strains, but it exhibited a good bactericidal effect without inhibition, against its homologous strain. Another

TABLE 8

BACTERICIDAL TESTS WITH			COMPLEMENT DOSE	TITER OF THE TWO FUNCTIONS	SERUM AGAINST							
					B. dysenteriae					B. typhosus	Vibrio cholerae	B metacoli
Species	Group	Strain			Normal rabbit serum	Rabbit 100, (Shiga), immunized from January 26, 1916, to April 11, 1916, with 12 loopfuls intravenously	Rabbit 90, Group IIA, immunized from September 2, 1915, to December 17, 1915, with 30 agar cultures intravenously	Rabbit 104 F. 31, group II, immunised from February 2, 1916, to March 22, 1916, 4½ cultures intravenously	Rabbit 99, group III, immunised from January 17, 1916, to April 17, 1916, 7 cultures intravenously	Rabbit 91, type I, immunised from October 2, 1915, to January 12, 1916, 15 agar cultures intravenously	Rabbit 101, (cholerae), immunised from January 26, 1916, to March 22, 1916, 3½ cultures intravenously	Rabbit 118, Metacoli, immunised from September 11, to November 21, 1916, 9½ agar-slant cultures intravenously
B. dysenteriae	I	Shiga	0.01	Inhibitory	0	0.0063	0	0	0	0		
				Bactericidal	0.0004	0.0000032	0.0002	0.0002	0.0002	0.0032		
	11	Danish I	0.04	Inhibitory	0	0	0.05	0.0125	0	0		
				Bactericidal	0.0008	0.0001	0.0000016	0.0000032	0.0008	0.0008		
		F. 52	0.04	Inhibitory	0	0	0.05			0		
				Bactericidal	0.0063	0.0001	0.0000063			0.0008		
		F. 29	0.02	Inhibitory	0	0.0032	0	0.0004	0	0		
				Bactericidal	0.0016	0.0000025	0.00005	0.0000016	0.0016	0.0016		
		F. 31	0.02	Inhibitory	0	0.0125	0	0.0032	0	0		
				Bactericidal	0.0008	0.000025	0.000025	0.0000016	0.0008	0.0016		

B. dysenteriae	III	F. 41	0.01	Inhibitory		0			0.0063			
				Bactericidal	0	0.0125	0		0.0002	0		
		F. 40	0.01	Inhibitory					0.0032			
		F. 40	0.01	Bactericidal	0				0.0002			
		Danish III	0.01	Inhibitory		0			0.0032			
				Bactericidal	0	0.0125	0		0.00005	0		
B. typhosus........................			0.02	Inhibitory	0	0		0	0	0		
				Bactericidal	0.05	0.0125	0	0.025	0.025	0.000025		
Vibrio cholerae.....................			0.04	Inhibitory							0.0032	
				Bactericidal							0.0001	
B. metacoli (Morgan I)..............			0.01	Inhibitory								0.0002
			0.01	Bactericidal								0.0000032

typhoid serum; however, showed both inhibiting and bactericidal action against this very same strain.

The cholera and metacolon sera showed strong inhibition and a moderate bactericidal effect against their homologous strains.

On the whole these tests show that the inhibiting effect is chiefly exhibited toward the homologous strains, but that is also seen, though less frequently, with the closely related strains.

4. Factors influencing the appearance of the phenomenon

On the basis of the results presented in table 3, we can examine the importance of the various factors involved in the bactericidal test with reference to the origin and growth of the inhibiting phenomenon. These factors are the antigen, the bactericidal immune serum; i.e., (the amboceptor) and the complement. As to the antigen, it seems that the very easily dissolved strains have a special faculty of giving rise to the inhibiting antibodies, while the strains that are more resistant to the serum bodies possess that faculty in only slight degree.

In the immunization of the animals it does not seem to matter much whether very large or smaller doses of antigen has been used. It must, however, be mentioned that all of these immunizations, in reality, have been carried out with considerable doses and, as a rule, by intravenous injection. Between 1 and 10 agar slant cultures have been used at a time. The bacteria, previous to their injection, were always suspended in physiological saline solution and heated for one hour at 60°C.

Rabbits, as a rule, weigh one twenty-fifth as much as a full-grown man; hence the proportional amounts of the bacteria for the average man, would be 25 to 250 agar slant cultures. It is clear that these doses represent many more bacteria than ever occur in man during a natural infection. It is possible that this is one of the reasons why Brekke found the phenomenon of inhibition so seldom; that is, in only 22 per cent of the sera of typhoid patients.

The dependence of the phenomenon of inhibition on the specific relationship between the antigen and the antibodies of the immune serum has been pointed out. Furthermore, it has been shown that normal sera lack the inhibiting function entirely. Hence it is not reasonable to suppose that the inhibition could be due to anything in the treatment of the serum after it has been obtained from the animal. However, the experiment was carried out to see whether the inhibiting function is influenced in any way by difference in the treatment of the serum.

A Shiga serum was tested in the fresh condition, then dried in a desiccator. The serum powder was then dissolved, partly in distilled water, partly in normal saline. All the tests showed the same inhibiting action of the serum.

Further, the effect of heating of the serum (one-half hour at 56°C.) on the inhibiting phenomenon was, also, examined and it was found to be nil; the inhibition was the same in the active and in the inactive immune serum, provided that the dose of complement added in the two tests was the same. The phenomenon is consequently not due to nor affected by any alterations in the serum after the bleeding of the animal.

5. Appearance of the inhibition during the immunization

The tests recorded in table 3 showed that the phenomenon of inhibition is just as specific as the bactericidal action. It seemed probable, therefore, that the first appearance and the growth of the phenomenon could be traced during the immunization. This was done in experiments presented in table 4.

It is seen that the inhibiting phenomenon, which is absent in the normal sera, appears after the first injection, and becomes more pronounced during the immunization. In the last test made four weeks after the last injection, the inhibiting as well as the bactericidal action is a little reduced.

6. Relation of the phenomenon to the bactericidal power of the serum

It is apparent that the inhibiting action as well as the bactericidal one is a result of the immunization, but this conclusion does not justify the supposition entertained by Neisser and Wechsberg and by Lipstein that both of these functions are exercised by the same antibody—the bactericidal amboceptor.

TABLE 4

Antigen B. dysenteriae of group II (two different strains employed)

	SERUM OF RABBIT 109	SERUM OF RABBIT 117
Before treatment	1* =0 B. =0.0063	1 =0 B. =0.0032
One week after first injection	1 =0.025 B. =0.000025	1 =0.0125 B. =0.0000008
One week after second injection		1 =0.0032 B. =0.0000008
One week after third injection	1 =0.0032 B. =0.0000063	
Four weeks after fourth injection		1 =0.0063 B. =0.0000032

*1 = Inhibiting action; B. = bactericidal action.

If, namely, the bactericidal titer (B) is taken as an indication of the amount of amboceptors present, this and the inhibiting titer (1) must stand in a direct proportion to each other, if these two functions be carried by the same antibody. However, such is not the case, as an analysis of the results presented in table 4 shows. With serum 109 the proportions were:

After the first injection..........I: B = 0.025 : 0.000025 = 1: 0.001
After the third injection........I: B = 0.0032: 0.0000063 = 1: 0.002

With serum 117 the proportions were:

After the first injection.........I: B = 0.0125: 0.0000008 = 1: 0.000064
After the second injection........I: B = 0.0032: 0.0000008 = 1: 0.00025
After the fourth injection.......I: B = 0.0063: 0.0000032 = 1: 0.00052

Thus, it is obvious that the proportion between I and B is *not* a fixed factor. This is demonstrated even better if we put together all the results of the bactericidal reactions and reduce the I function to 1 while the B function is proportionally reckoned out. This has been done and the results are presented in table 5.

TABLE 5

STRAIN	INHIBITION	BACTERICIDAL ACTION	I : B
Shiga	>0.1	0.0032	?
	>0.1	0.0002	?
	>0.1	0.0004	?
	0.0125	0.0000032	1:0.00025
	0.0063	0.0000032	1:0.00052
	0.0032	0.000000025	1:0.0000078
Danish I	>0.1	0.0008	?
	>0.1	0.0001	?
	>0.1	0.0000063	?
	0.05	0.0000016	1:0.000032
	0.0125	0.0000032	1:0.00025
	0.0063	0.0000025	1:0.0038
F 52	>0.1	0.0063	?
	>0.1	0.0008	?
	>0.1	0.0001	?
	0.05	0.0000063	1:0.000126
	0.0125	0.0004	1:0.032
F 29	>0.1	0.0016	?
	>0.1	0.00005	?
	0.0125	0.0001	1:0.008
	0.0032	0.000025	1:0.0078
	0.0004	0.0000016	1:0.004
	0.0001	0.0000016	1:0.016
F 31	>0.1	0.0016	?
	>0.1	0.0008	?
	>0.1	0.000025	?
	0.0125	0.0001	1:0.008
	0.0125	0.000025	1:0.002
	0.0032	0.0000016	1:0.0005
	0.0008	0.0000025	1:0.03
F 41	0.0063	0.0002	1:0.03
F 40	0.0032	0.0002	1:0.06
Typh. 1	>0.1	0.000025	?
	0.0032	0.00005	1:0.015
Vib. cholerae	0.0032	0.0001	1:0.03
Bac. metacoli	0.0002	0.0000032	1:0.016

It is seen that the bactericidal titers vary in their proportion to the inhibiting titer (taken as 1) from 0.06 to 0.0000078 This would be quite impossible if these two functions were due to the same antibody, because, in that case, there would have to be a constant ratio between the two functions.

The variations in the ratio 1: B confirms Brekke's statement that a high bactericidal titer does not *eo ipso* convey a high inhibiting titer, and vice versa. By these findings we are forced to reject the theory of the significance of the bactericidal amboceptor for the inhibition. Since, however, the inhibition arises simultaneously with the bactericidal function and is quite specific, the former must owe its origin to specific antibodies other than the amboceptor. How these antibodies must be supposed to act and how they originate, will be presently dealt with.

TABLE 6

COMPLEMENT	INHIBITION	COMPLEMENT	INHIBITION
0.05	0.05	0.02	0.0016
	0.025		0.0001
0.04	0.05	0.0125	0.0016
	0.025	0.01	0.0125
0.03	0.0125		0.0002
	0.0032	0.009	0.2
0.025	0.0125		0.025
	0.0032	0.007	0.0032
	0.0008		0.0032
0.02	0.0125	0.005	0.0125
	0.0032		0.0063—0.0032

7. *Relation of inhibition to complement*

If we examine the relation between inhibition and the dose of complement, we find that this seems to be of a more stable character. From the material of the bactericidal tests made in this institute, it is obvious that the very high inhibiting titers are to be found in tests where small doses of complement have been employed, while the tests in which large doses of complement have been used, usually show low titers. This will be seen quite clearly from table 6.

Still better we will see the relation between the dose of complement and the inhibition in some tests made upon the same serum with different doses of complement. The tests were made at different times and not for the purpose of showing this fact.

Test A. Antigen: dysentery Shiga.

Immune serum: produced against dysentery Shiga, dose 0.1 = 0.0008.

Complement: guinea-pig serum (doses: 0.005 and 0.02).

C = 0.005	C = 0.02
1 = 0.0032	1 = 0.0125
1:C = 1.: 1.56	1: C = 1.:1.60

Test B. Antigen: dysentery, (group iii) (F. 79).

Immune serum: produced against F.79 (doses: 0.2 – 0.0032).

Complement: guinea-pig serum (doses: 0.009 and 0.08).

C = 0.009	C = 0.08
1 = 0.025	1 = 0.02
1:C = 0.36	1:C = 1:0.40

Test C. The following test is made directly to demonstrate how 1 varies with C. The protocol of this test is presented in table 7.

Antigen: *B. typhosus.*

Immune serum: Produced against *B. typhosus.* Doses: 0.0063, 0.0125, 0.025, and 0.05.

Complement: Guinea-pig serum. Doses: 0.4 – 0.0125.

C = amount of the normal guinea-pig serum used.

1 = the "inhibiting titer."

These three tests very clearly show how the titer of the inhibition varies according to the dose of complement. This relation between inhibition and complement, indeed, is so close that, in the same serum, we find an absolutely stable ratio between the smallest dose of the serum that gives the inhibition and the employed dose of complement. It is clear, however, that we can not expect in all cases to find the same ratio between I and C, as a fixed amount of a complement-bearing serum does not always have the same functional value.

The establishment of a fixed ratio between I and C is further prevented by the fact that the inhibiting power of each serum

TABLE 7

COMPLEMENT	IMMUNE SERUM			
	0.0063	0.0125	0.025	0.05
	Number of colonies			
0.4	5	10	10	20
0.2	20	10	10	20
0.1	20	5	5	2000
0.05	20	20	Many 1000	
0.025	1000			
0.0125				
1:C	1:2		1:2	1:2

TABLE 8

GROUP	STRAIN	TITER OF INHIBITION	DOSE OF COMPLEMENT	RATIO I:C
I............	Dys. Shiga	0.0032	0.03	I: 8.1
		0.0005	0.005	I: 10.0
		0.0032	0.005	I: 1.56
		0.0125	0.02	I: 1.60
II............	0.36	0.0125	0.025	I: 2.0
		0.05	0.05	I: 7.0
	F. 11	0.0125	0.03	I: 2.4
		0.025	0.04	I: 2.0
	F 52	0.05	0.04	I: 0.8
	Danish I	0.025	0.04	I: 1.6
	F 29	0.0001	0.02	I: 200
		0.0002	0.01	I: 50
		0.0016	0.0125	I: 7.8
		0.0032	0.03	I: 9.3
III...........	F 41	0.0125	0.01	I: 0.8
		0.0032	0.007	I: 2.2
		0.0032	0.006	I: 1.9
Typhoid.......	Typh. I	0.0032	0.02	I: 6.25

is different and varies from time to time. It is only possible to show the relation between I and C in each serum. The ratio I: C will then be the same in all tests made with the same immune serum and the same complement.

As this ratio is of a quite stable character, it looks as if a certain dose of immune serum inhibits the action of a certain dose of complement and that the larger doses of serum can inhibit the action of correspondingly larger doses of complement. In table 8 some inhibition titers are put together with the employed dose of complement. The tests were made at different times and with different complements; consequently, we cannot expect to find the ratio I: C so constant as in tests with the same serum and complement.

The table shows that the value of the different immune sera reckoned according to their faculty of inhibiting the action of the complement is very different. Thus, while one unit of the strongest inhibiting serum checked the action of 200 units of complement, the same amount of the weakest serum could prevent the function of only 0.8 units of complement. It is seen, furthermore, that in three instances I: C is I: 10 or more, four times it is between I: 10 and I: 5, eight times it is about I: 2 and three times it is I: I or less. Thus, in the majority of the cases, we find that one dose of immune serum is capable of preventing the function of two doses of complement as one of the demonstrated tests shows.

The foregoing results make it obviously incorrect to compare the inhibition titers of various sera, unless due regard is paid to the employed dose of complement.

8. *Inhibition in normal sera*

Since the inhibition had been found to be dependent, to such an extent, upon the employed dose of complement, it was natural to think that normal sera also might show the phenomenon, if only the dose of complement was small enough or the dose of serum large enough.

This consideration applies especially to the tests where normal rabbit sera was found to show a quite high bactericidal action against the dysentery strains of group I and II. It seems possible that these sera would have shown the inhibition phenomenon in higher doses.

In table 9 are presented the protocols of some tests with three normal rabbit sera in high doses.

Rabbits I and II in these tests show a strong inhibition down to the dose 0.25 cc., while rabbit III shows a weak inhibition only in the dose 0.75 cc. Thus, it seems that even wholly normal inactivated serum can inhibit the function of complement, when large doses are employed. Since this non-specific inhibition is found only with much larger doses of serum than are used for the bactericidal tests, there is no occasion on account of this phenomenon to doubt the specificity of the corresponding phenomenon in the immune sera.

TABLE 9

SERUM	RABBIT I	RABBIT II	SERUM	RABBIT III	
	Dys. Shiga C* = 0.008	Dys. II (F. 31) C = 0.04.		Dys. III (F. 79) C = 0.04	B. typhosus C = 0.04
	Colonies			Colonies	
1.0	∞	∞	0.75	1000	1000
0.5	∞	∞	0.25	400	100
0.25	Many 1000	Many 1000	0.125	100	100
0.125	About 1000	50	0.063	50	50
0.063	10	5	0.032	30	50
0.032	10	5	0.016	30	20

* C = Complement.

Whether this normal inhibition is of the same nature as the specific one, is an open question. It seems likely that this need not be the case. In fact the doses of normal serum that give inhibition are so large, that it is possible that the inactivated serum taken as a solution of albumin absorbs the complement in the manner of colloids. It may also be supposed that a concentrated, inactivated serum contains so many complementoids that these become more numerous than the effective complement, and so inhibit the function of the latter.

A study of the preceding experiments makes it clear that we can expect to find a zone in the dilutions of serum between the concentrated and the I: 10 dilution, where the inhibition phenomenon can occur without its being possible to decide whether it is specific or non-specific. In sera diluted more than I: 10 we should most likely be out of this zone of inhibition. When we begin the bactericidal tests with the doses 0.1 or 0.05 cc., we will, therefore, usually not be troubled by the non-specific inhibition. However, it has often happened that some hundreds (200–300–500) of colonies have grown up in the largest dose of serum used. This has not been taken as an inhibition, but has been looked upon as an accidental occurrence. It seems possible, now, that this was something like the tip of the tail of the inhibition, and that this phenomenon might have been found in full strength had only higher doses of serum been employed. We would then, however, have been incapable of deciding whether this was a specific or a non-specific inhibition.

9. *Inhibition in active, immune sera*

The ratio between I and C, referred to above, unveils another and very peculiar action of immune sera. It must be supposed that the immune serum that is able to inhibit the function of a foreign complement is able, also, to bring about the same action against the complement of the immune serum itself. If, now, we suppose that the test of an immune serum shows the most frequent ratio between I and C; namely 1:2, one unit of the serum taken as an immune serum should be able to check the function of two units of the same serum taken as a complement. Consequently, 0.5 cc. of the immune serum will be able to inhibit the function of the complement contained in 1 cc. of the same serum. The conclusion must be, that such a serum never can show any bactericidal action against its homologous strain of bacilli, whatever amounts of the active serum are employed without added foreign complement. This will further lead to the assumption that all the sera with a high titer of inhibition must have lost the faculty of bactericidal action that they had before the immunization. If this assumption proves to be true, we will see that sera with some normal bactericidal effect will not grow more effective with regard to total bactericidal power as the immunization proceeds, but they will lose all the effect that they had before. And if this is so in the tests *in vitro*, it is most likely the same *in vivo*, because here the serum or plasma never is present in diluted condition or with added foreign complement, but it is always concentrated. In fact, the results presented in tables 10 and 11 show that the assumption is correct. In these tables are demonstrated the results of tests carried out with the serum of a rabbit that had been immunized first against *B. typhosus* and then against *B. dysenteriae*, Shiga. Before the treatment the serum of the rabbit very rapidly and easily dissolved typhoid as well as dysentery bacilli, while this faculty was completely lost after some injections of antigen.

During the immunization with typhoid bacilli, tests with dysentery bacilli were carried out to demonstrate that the action of the serum against these latter bacilli was unaltered and although this latter function remained undiminished, the bactericidal effect against the typhoid bacilli was completely lost.

During the immunization with the bacillus of Shiga, the same control tests were carried out with a strain belonging to dysentery group III. This group III stain remained very sensitive to the serum, while the serum lost all its former effect against the bacillus of Shiga.

The strong specificity of the phenomenon is demonstrated in these tests.

As regards the typhoid bacilli, tests were carried out one year after the last injection. The serum then had again acquired its former faculty of total bactericidal effect. The inhibiting antibodies had diminished so much that they only could act in the dose of 0.1 cc. with 0.02 cc. of complement. According to the proportion $I:C = 0.1 : 0.02 = 1 : \frac{1}{5}$, it is seen that one unit of the serum as an inhibiting serum inhibits the action of only $\frac{1}{5}$ of the complement at hand. Consequently, there is left $\frac{4}{5}$ of the complement to bring about the bactericidal action.

TABLE 10

Rabbit 180

Immunisation against typhoid bacilli

Injections: January 9, 1918, $\frac{1}{10}$ loopful
January 18, 1918, $\frac{1}{5}$ loopful
January 30, 1918, $\frac{1}{3}$ loopful
February 8, 1918, 2 loopfuls
February 21, 1918, 2 loopfuls
March 5, 1918, 3 loopfuls

SERUM DOSES	TESTS IN ACTIVE SERUM; NUMBER OF COLONIES											
	Test before immunisation		Test January 25		Test February 28		Test March 9	Test March 16		Test in inactive serum, C = 0.02, March 16	Test March 21, 1919	Test in inactive serum, C = 0.02, March 21, 1919
	Typhoid	Dysentery III	Typhoid	Dysentery III	Typhoid	Dysentery III	Typhoid	Typhoid	Dysentery I	Typhoid	Typhoid	Typhoid
0.4	0	0	0	0			Many 1000					
0.2	0	0	0	0			Many 1000	∞	9		50	
0.1	0	0	0	0	∞	8	1000	Many 1000	6	Many 1000	50	∞
0.05	0	0	0	0	Many 1000	10	Many 100	Many 1000	16	Many 1000	50	100
0.025	0	0	0	0	Many 1000	12	100	1000	100	Many 1000	100	100
0.0125	20	0	0	0	1000	10	200	1000	Many 1000	1000	Many 1000	100
0.0063	100	0	∞	0	1000	20	Many 1000	∞	∞	1000	∞	Many 100
0.0032	Many 1000	0	∞	∞	Many 100	∞	Many 1000	∞	∞	50	∞	Many 100
0.0016	Many 1000	20	∞	∞	Many 1000	∞	Many 1000	∞	∞	30	∞	Many 100
0.0008	∞	500	∞	∞	∞	∞	Many 1000	∞	∞	1	∞	Many 1000
0.0004	∞	1000	∞	∞	∞	∞	Many 1000	∞	∞	1	∞	Many 1000
0.0002	∞	∞	∞	∞	∞	∞	Many 1000	∞	∞	0	∞	∞
0.0001												∞
0.00005												∞
0.000025												∞

TABLE 11

Rabbit 180; immunization against dysentery bacilli (group I (Shiga)

Injections: March 14, 1918, 1/100 loopful; October 4, 1 culture; January 15, 1919, about 2 cultures
May 3, 1918, 1/50 loopful; October 8, 1 culture; January 29, 1919, about 2 cultures
May 6, 1918, 1/10 loopful; October 28, 1 culture; February 11, 1919, about 2 cultures
May 13, 1918, 1/5 loopful; November 9, 1 culture; February 28, 1919, about 2 cultures
June 5, 1918, 1/2 loopful; December 5, 1 culture; March 13, 1919, about 2 cultures
July 1, 1918, 1 loopful; December 31, 1 culture; March 19, 1919, about 2 cultures

SERUM DOSES	TESTS IN ACTIVE SERUM; NUMBER OF COLONIES										
	Tests before immunization		Test May 12	Test June 5		Test in inactive serum, C = 0.008, in added complement	Test October 1, 1918		Test November 18	Test March 26, 1918	Control test in normal rabbit serum
	Dysentery I	Dysentery II	Dysentery I	Dysentery I	Dysentery III		Dysentery I	Dysentery III			
0.4			∞	∞	0						
0.2		9	∞	∞	0		3000	0	∞	∞	
0.1	30	6	Many 1000	∞	0		2000	2	∞	∞	20
0.05	30	16	Many 1000	∞	0	∞	1000	50	∞	∞	100
0.025	50	100	Many 1000	∞	6	∞	1000	100	∞	∞	1000
0.0125	Many 100	Many 1000	Many 1000	∞	50	∞	Many 1000	50	∞	∞	∞
0.0063	Many 1000	∞	About 1000	∞	500	∞	∞	Many 100	∞	∞	∞
0.0032	∞	∞	Many 1000	∞	∞	∞	∞	∞	∞	∞	∞
0.0015	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞
0.0008	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞
0.0004	∞	∞	∞	∞	∞	Many 1000	∞	∞	∞	∞	∞
0.0002	∞	∞	∞	∞	∞	Many 1000	∞	∞	∞	∞	∞
0.0001						100					
0.00005						50					
0.000025						100					
0.0000125						Many 1000					
0.0000063						∞					
0						∞					

If we expect to find inhibition in active sera, I: C must be I: I or exceed this ratio.

As now the greater part of our immune sera show the proportion I: C = 1:2 or more, we must suppose that all these sera have lost their total bactericidal action during the immunization, and this is not only the case with the sera from rabbits. On the contrary, 34 out of 64 sera from typhoid patients (Brekke) showed so high inhibiting titers that they no doubt must have been robbed of all their total bactericidal action. The same has been found by the author as regards sera from dysentery patients.

If we wish to transfer the results of these experiments *in vitro* to the conditions *in vivo*, we must conclude that blood fluid from immune organisms (if it is supposed that this is identical in its action with the serum) so far from being more bactericidal than the same in the normal state, loses its bactericidal effect during the immunization or the disease. Consequently, the bactericidal action of the serum can not play any part in the stable immunity after a disease, or after an artificial infection. The experiments show clearly that the immune animals, whose serum cannot kill the bacteria in question, tolerate the inoculation much better than the normal animals, whose serum is able to kill large amounts of bacteria. And we have no reason to believe that the patients that show a high degree of inhibition are not quite as immune as those, whose serum shows a high degree of bactericidal effect. If the results of the test tube experiments are applicable to the conditions in the living organism, it would appear probable that the animals with the strong inhibition tolerate the inoculations better than the normal animals just on account of the acquired faculty of inhibition. The effect of an injection of bacilli into the organism of a normal animal with bactericidal action, may be that the bacilli will be very rapidly dissolved and their endotoxins made free to intoxicate the organisms, if the dose of bacilli was large enough. This will, perhaps, not be the case in an immune organism with a well developed inhibiting action. Here the bacilli will either not be dissolved in the blood stream or their solution will go on more slowly and other immune forces can come into play and deal with the bacilli. Consequently, the endotoxins are not made free or at any rate they are liberated very slowly, and the animal is saved from the intoxication that kills the normal animal.

Since most normal sera in active, concentrated condition are able to dissolve or kill the bacteria here dealt with, but lose their faculty (or acquire the faculty of inhibition) during the disease or the immunization, it is probable that the specific inhibiton plays a far more dominant part in the protection of the organism during the infection than is the case with the bactericidal action of the serum. On the other hand we cannot consider the inhibiting action as any protection against becoming infected, and the phenomenon, therefore, can hardly be of any importance to the lasting immunity after the disease or inoculation.

We have considered the relation between inhibition and complement without setting up any definite theory as to its explanation. We have also expressed the idea that the inhibiting serum interferes with or checks the function of the complement.

The question now to be answered is this:

What has happened to the complement?

That it is not fixed to a surplus of amboceptors, as held by Neisser and Wechsberg, must be considered beyond doubt. Consequently, we must consider the following possibilities:

1. The complement may for some reason be hindered in attaching itself to the bacteria and amboceptors and remains free in the fluid.

2. The complement may be fixed to the amboceptor in the normal manner, but unknown antibodies render the bacteria invulnerable.

3. The complement may be fixed to other antibodies than the bactericidal ones, and cannot, therefore, be available for the bactericidal reaction.

1. To test the first possibility the following experiment was carried out. To an active anti-Shiga serum showing inhibition homologous bacilli were added and the mixture was incubated for 3 hours at 37°C. The bacilli were separated from the fluid

by centrifugation and the absorbed serum was tested as to its total bactericidal action against a strain of dysentery III, which is very sensitive to the normal serum.

Simultaneously, a control test was made in active, not absorbed serum from the same animal.

Result: In the absorbed serum, no bactericidal effect.

Result: In the not absorbed serum: B = 0.0063.

It is seen that complement does not remain free in immune serum during its contact with the related bacteria in the zone of inhibition.

2. The second possibility is considered in the theory of Sormani. It supposes that the surface of the antigen is altered by the inhibiting antibodies in such a way that it is rendered invulnerable. If this be the case, it should be possible to show this acquired invulnerability, when the bacteria that have been in contact with an inhibiting serum, are brought into another, not inhibiting, solution.

The following experiment was carried out to test this supposition.

Bacteria were kept in contact with the specific antiserum—F.79—(I = 0.025; C = 0.009) in the ratio 1/8000 oese to 0.1 cc. of serum in a volume of 1 cc. After the mixtures had stood for different periods of time at different temperatures, the bacteria were separated from the fluid by centrifugation and by washing in sterile saline solution and compared with the untreated bacteria of the same strain as to their susceptibility to the normal bactericidal action of fresh guinea-pig's serum. As some growth of the bacteria took place during their contact with the inhibiting serum corresponding dilution of the bacterial sediment had to be made before the rest in the guinea-pig's serum..

The results of the experiment are presented in table 12.

It is seen that the treated bacteria were killed as easily as the untreated ones, consequently, during their contact with the inhibiting serum they had not acquired any faculty that rendered them invulnerable to the antibodies.

3. The complement may be supposed to be absorbed by antibodies of another nature than the bactericidal ones. The con-

nection between these antibodies and the complement may be thought to take place with the production of a precipitate that can be brought down as a sediment. Also, a union may be assumed to occur between antigen and antibodies that remains in solution, and for this reason cannot be removed from the fluid by mechanical means. In the first case it must be possible to remove the inhibiting antibodies after addition of antigen to the serum and later centrifugation of filtration of the fluid. The antigen then must be supposed to absorb the inhibiting antibodies from the serum and thus remove them from the solution. In the other case, the removal of the antigen cannot be thought to rob the serum of its inhibiting faculties, as the soluble connection between antigen and antibodies will remain in the fluid after centrifugalization or filtration and be capable of absorbing complement when this is added.

TABLE 12

GUINEA-PIG'S SERUM	A. FOUR HOURS CONTACT IN INCUBATOR	B. FOUR HOURS CONTACT IN THE COLD	C. TWENTY.FOUR HOURS CONTACT IN INCUBATOR	D. NOT TREATED
	Colonies			
0.5	3	0	0	0
0.25	2	0	0	2
0.125	5	2	50	0
0.063	10	1	5	4
0.032	Many 100	1	4	5
0.016	Many 1000	100	50	∞
Control	∞	∞	∞	∞

To test these possibilities, the following experiments were carried out:

1. To an anti-Shiga serum was added an emulsion of living Shiga bacilli in great excess. After two or three hours in the incubator at 37°C., the bacteria were removed in the centrifuge and new bacteria were added. This procedure was repeated eight times during thirty hours. No agglutination could be seen after the last addition of bacteria.

In the serum thus absorbed, bactericidal tests were carried out as well with a dose of complement suitable for the activation

(0.005) of the serum as with a dose that itself had a bactericidal action (0.02). Simultaneously, control tests were carried out with untreated serum. The results of this experiment are presented in table 13.

It is seen that the prolonged contact of the immune serum with the antigen caused diminution of its inhibiting action although all of its bactericidal property had been removed.

Another anti-Shiga serum was treated in the following manner.

2. One part of the serum was diluted 1:5 and mixed with a great excess of Shiga bacilli; this mixture was shaken at room temperature. After four hours the bacilli were removed in the centrifuge and the supernatant fluid was mixed with fresh bacteria. This was repeated every fourth hour during forty-eight hours (nights excepted). After the last centrifugation the fluid was divided in two halves. One portion was heated at 56°C. for one hour and again centrifugalized. The serum was then quite clear and showed no growth in a control test. The other half was filtered through a Berkefeld filter. Control culture showed no growth. As a control test some non-absorbed serum was filtered.

TABLE 13

IMMUNE SERUM	A. COMPLEMENT = 0.02		B. COMPLEMENT = 0.005	
	Untreated serum	Absorbed serum	Untreated serum	Absorbed serum
	Colonies			
0.1	∞	∞	∞	∞
0.05	∞	∞	∞	∞
0.025	∞	∞	∞	∞
0.0125	∞	∞	∞	∞
0.0063	About 100	About 100	∞	∞
0.0032	0	0	About 100	∞
0.0016	0	0	0	∞
0.0008	0	0	0	∞
Complement control	0	0	∞	∞

With these three sera bactericidal reactions were carried out. As complement was used a dose of fresh serum that was capable of bringing about bactericidal action by itself. The results of this experiment are presented in table 14.

It is seen that here again prologned contact of the inhibiting serum with the homologous bacteria resulted in no diminution of its inhibiting power whether the bacteria are removed with centrifugation or by means of filtration.

Another portion of the same serum was treated in the following manner:

Three cultures (surface 7 by 15 cm.) of Shiga bacilli emulsified in normal saline solution (30 cc.) were heated at 60°C. for one hour, the emulsion was then centrifugalized, the bacilli washed in normal saline and afterwards mixed with the serum diluted 1:10; after being shaken at room temperature for 24 hours, the mixture was centrifugalized and a new bacterial emulsion was added to the supernatent fluid. This procedure was repeated four times.

TABLE 14

SERUM	A. ABSORBED, HEATED SERUM	B. ABSORBED, FILTERED SERUM	C. UNHEATED, FILTERED SERUM
	Colonies		
0.1	∞	∞	∞
0.05	∞	∞	∞
0.025	About 2000	Many 1000	About 1000
0.0125	1000	1000	200
0.0063	200	50	3
0.0032	50	20	5
0.0016	10	10	0
0.0008	5	2	10
0.0004	10	10	10
0.0002	5	50	10
0.0001	50	20	10
Complement control	5		
Serum control (0.1 Serum without bacilli)	0	0	0

With this treated serum tests are made in the ordinary manner. Complement was employed in amount (0.025 cc.) that was itself capable of producing bactericidal effect. The results of this experiment are presented in table 15.

It is seen that the prolonged contact of the inhibiting serum with the killed and washed bacteria failed to reduce its inhibiting power.

Similar tests were made with the same serum absorbed at a temperature of 5°C. with living bacilli during six days. The serum thus treated showed a little more inhibition than the untreated serum.

Likewise the same tests were carried out with a serum produced against *B. dysenteriae* group III (twenty-four hours contact with living bacilli). Also in this treated serum, a rather stronger inhibition was noted than in the untreated serum.

TABLE 15

SERUM	A. ABSORBED SERUM	B. UNTREATED SERUM
	Colonies	
0.05	∞	∞
0.025	∞	∞
0.0125	∞	∞
0.0063	Many 1000	Many 1000
0.0032	About 200	About 500
0.0016	50	50
0.0008	10	10
0.0004	10	3
0.0002	5	6
Complement control	10	
Cerum control (0.1 serum)	0	
Control on bacilli used	∞	

Thus it has not been possible to remove the inhibiting effect from the sera examined through absorption with the homologous antigen.

Objection may possibly be made to the first experiment, that living bacilli may have been left in the serum, as no control test was made to exclude this possibility. The dose of active serum, however, was so large (0.02 cc.), that it should easily have been able to kill the small number of bacilli that might have been left after the centrifugation, as it killed all the bacilli in the control test. This objection cannot be made against the last two experiments, as the serum here was found to be sterile before and during the test.

It has thus been impossible to free the sera of the inhibiting action through absorption with homologous antigen, as carried out in this investigation. From the above mentioned experiments we must conclude that the inhibiting antibodies cannot be fixed to the bacillary bodies or to sedimenting antigen and be removed from the serum with these; also, that the inhibiting antibodies are quite different from the bactericidal amboceptors, as the inhibiting action may be found in sera that have been deprived of all bactericidal power.

DISCUSSION

If, on the basis of the foregoing experiments and results, we wish to try to form an opinion as to the nature of the inhibiting antibodies and the value of the different theories concerning them, it may be useful to consider the various possibilities in the light of Ehrlich's side-chain theory and to compare these possibilities with the facts brought forth by these experiments.

1. Neisser and Wechsberg did not assume any special inhibiting antibody, but thought that the great surplus of bactericidal antibodies made the action of the complement impossible. This conception is graphically represented in figure 1.

This theory is made impossible by the following facts:

a. There is no relation between the bactericidal titer and the inhibiting titer.

b. There may be found immune sera with a high bactericidal titer, but without any inhibiting action.

c. The inhibition can still be found in sera from which the bactericidal amboceptors have been removed by absorption with homologous antigen.

2. The idea that during the active imunization of an animal an antibody is produced that is capable of rendering the bacteria invulnerable to the action of the bactericidal amboceptor-complement complex although the latter is not prevented from combining with the bacteria. This idea is contained in the theory of Sormani. It is represented graphically in figure 2. This assumption is rendered untenable by two facts brought out

in the present investigation, namely, (1) the hypothetical antibody cannot be absorbed from the inhibiting serum and (2) the bacteria that have been in contact with an inhibiting serum are not less vulnerable to the bactericidal amboceptor-complement complex than are untreated bacteria.

3. The hypothesis that the inhibiting antibodies act by attaching themselves to the antigen, thereby hindering the union of the latter with the antigenophil group of the bactericidal amboceptor, is represented graphically in figure 3.

This hypothesis is excluded by the fact, demonstrated above, that the bactericidal amboceptors can be absorbed from the serum by the bacteria in the presence of the inhibiting antibodies, and that the latter actually do not become attached to the bacteria at all but remain free in the fluid.

4. During the immunization it is conceivable that an antibody of the nature of an antiamboceptor is produced. By fixing itself to the antigenophil group of the amboceptor; this antiamboceptor might hinder the connection between amboceptor and antigen. The latter would, in this case, remain free and the bactericidal action could not take place. This conception is represented graphically in figure 4. This theory necessitates that the combination amboceptor-antiamboceptor absorbs the complement with greater avidity than the combination amboceptor-antigen. The bactericidal amboceptors namely, are at hand in a far greater amount than the supposed antiamboceptors (I. gives always a lower titer than B.) and the amboceptor-antigen compound therefore would absorb the greater amount of complement, if the avidity in both cases were the same. But if an antiamboceptor connected with its homologous amboceptor could absorb all of the complement, the nature of the antigen would play no part in the inhibition, and the inhibition would thus take place against any bacteria tested in an inhibiting serum. As this is not the case, the theory cannot be accepted.

5. Conceivably, the production of antiamboceptors against the complementophil group of the amboceptor (complementoids) may take place during the immunization. Such antibodies would not prevent the union of the bactericidal amboceptor

with the bacteria, but they would prevent the subsequent cöoperation of complement. According to this view, which is graphically represented in figure 5, bacteria after contact with an inhibiting concentration of immune serum should be invulnerable to the action of fresh active serum, since all of the receptors are supposed to be satisfied, moreover, an absorbed inhibiting immune serum should possess bactericidal power since, according to this view, its complement should remain unaltered in the fluid after the absorption. Since neither of these two conditions are met in actual experiment, the theory cannot be sustained.

The theory of Brekke must be dealt with under this last possibility. This theory must be rejected, as it is demonstrated that the inhibition takes place, also, in quite fresh and active sera.

6. Finally, it is possible that during the immunization specific antibodies are produced, which in connection with dissolved antigen, absorb complement with a greater avidity, so that the complement cannot effect its bactericidal action. Figure 6 represents this idea graphically.

This supposition necessitates that the titer of inhibition varies with the dose of complement, since a certain dose of inhibiting antibody must absorb a certain dose of complement. If the dose of complement is larger than that required by the inhibiting antibodies, we must suppose, that the rest of complement is made use of by the bactericidal antibodies. If, on the other hand, the dose of complement is very small it should be possible to show the existence of very small doses of inhibiting antibodies. Further, the bacteria that have been in contact with an inhibiting serum can be entirely vulnerable in a non-inhibiting serum, since nothing has happened that should diminish their vulnerability. On the other hand, the absorbed serum, according to this view, should lose all its former bactericidal action, as no free complement will be left in the solution.

All the above described experiments satisfy these claims, and this theory may therefore be accepted.

The specific inhibiting antibody thus acts like an anticomplement inasmuch as it hinders the function of the complement.

Since, however, it can act only in the presence of the homologous antigen, we must suppose that a combination of this antibody and the antigen absorbs the complement. We must, therefore, look upon the inhibiting antibody as being of the nature of an amboceptor, but not as an amboceptor that enters into union with the solid sedimented bacterial protein, since it has not been possible to remove the inhibiting antibody from the serum by absorption and centrifugalization or filtration. We must rather suppose that the inhibiting antibody in connection with dissolved antigen forms larger colloidal molecular complexes that absorb the complement at hand with great rapidity without the formation of a precipitate, which could be removed from the fluid. This explanation is the same as that used by Gengou and by Moreschi in their work on anticomplementary action. We have here, also, an explanation of the great similarity between the specific action of the mentioned inhibiting antibodies and the non-specific action of such complement inactivating bodies as bile, peptone, albumose, citrate, and oxalate solutions. The action of the latter bodies is non-specific; the complement is inactivated in any combinations whatsoever. The action of the inhibiting antibody, however, is dependent on the presence of the homologous antigen. But with this exception the action and the result of the action is the same in both cases; namely, the inhibiting of the function of the complement, and hereby the hindering of the bactericidal action.

It is possible that the examination of the physical colloidal conditions of the serum absorbed with its homologous antigen would bring us closer to the solution of the problem of the inhibition than this investigation has been able to do.

If we wish a working hypothesis as to the origin of the inhibiting antibodies, we may assume the following:

When antigen gets into the veins of man or animals, it will, partly at any rate, go into solution. The dissolved antigen will bring about the production of immune bodies against itself. Among those bodies will be found some that precipitate the dissolved antigen, and others that enter into combination with the antigen without producing a precipitate. This last combination is capable of binding complement and the antibodies concerned will, for this reason, appear as inhibiting antibodies.

Thus, the production of inhibition in a serum must be looked upon as a reaction of immunity just as agglutination, precipitation and the bactericidal action. It is possible that the inhibiting reaction might be employed as a diagnostic aid in infections with easily soluble bacilli.

The phenomenon of specific inhibition has hitherto been looked upon as a peculiar and paradoxical function of immunesera. Practically, however, it has not yet attracted any interest. It is nevertheless probable that the phenomenon of inhibition may play a part in the action of the therapeutic immune sera. This will, however, take place only in sera possessing bactericidal or bacteriolytic action which, in practice, means the antimeningococcic serum. The antimeningococcus serum is widely used and is considered to be of use in the treatment of meningitis. However, its therapeutic effect must be due to antibodies that do not need the coöperation of complement, because this function is absent in the stored serum, and the normal spinal fluid lacks complement. Under these circumstances the bactericidal function of the serum cannot be brought into action.

If the attempt should be made to improve the effectiveness of the antimeningococcus serum by the addition of active serum—say from the patient himself—it would be important, first to ascertain the titer of inhibition of the immune serum, if that property exists in such sera—a question which has not been investigated. If, for example, the ratio I: C were found to be 1:2, this would mean that the ordinary dose of the immune serum (25 cc.) would nullify the complementary action of 50 cc. of active serum. Hence, it would be necessary, under these circumstances, in order to secure the coöperation of complement, to inject, with the immune serum, *more* than 50 cc. of the normal serum. However, as the spinal column is not able to accommodate this large volume of fluid, it would be necessary to adopt the other alternative of injecting less of the immune serum than usual. For example if we choose to inject the usual volume of 25 cc., and if we wish to inject a mixture of immune serum and complement-bearing serum in which the ratio of the latter to the former is more than 2: 1, say 5: 1, we can use the following mixture:

	cc.
Immune serum	1.0
Fresh normal serum	5.0
Sterile saline solution	—19.0

It is likely that such a diluted mixture would be more efficacious than an undiluted one, as it might penetrate more easily into the meninges.

The necessity, in such procedure, of determining the ratio I: C for each specimen of immune serum, must be pointed out.

The possibility that the use of a strongly bactericidal mixture may cause an intoxication as the result of a sudden liberation of endotoxins in large amounts, should be borne in mind.

Where, on the other hand, sera are dealt with that are to be injected intravenously, the inhibiting action cannot, as a rule, play any part in the therapeutic effect. In such case there will always be a far greater amount of active serum at hand than the dose of inhibiting serum. Only if the serum has a very high titer of inhibition, may there be a slight possibility of inhibition. Thus if I: C were 1: 200, as in one of our dysentery sera, 10 cc. of inhibiting serum would nullify the complement in 2000 cc. of active serum, and in this case 20 cc. of inhibiting serum injected intravenously would produce an inhibiting mixture, as a grown up man has about 2500 cc. of blood fluid.

CONCLUSION

1. The inhibiting phenomenon of Neisser and Wechsberg is of a specific nature. It is to be found in active as well as in inactive sera; it develops during the immunization and can be found in a very high degree in dysentery immune sera. In active sera from immunized animals examined without the addition of foreign complement, the phenomenon presents itself as a complete abolition of the normal bactericidal action.

2. The inhibition is due to antibodies that arise during the immunization or during the natural disease. These antibodies

are not identical with the agglutinins, the bacteriolysins or the precipitins. They must be considered as specific antibodies, which combine with dissolved antigen to form molecular complexes, that have a marked tendency to absorb complement and to withdraw it from the bactericidal antibodies.

3. The titer of inhibition is directly proportional to the employed dose of complement. With a small dose of the latter, smaller doses of inhibiting antibodies can be demonstrated than with a larger dose of complement.

4. The inhibiting antibodies do not effect the bacteria themselves, nor can they be removed from the serum by absorption with an emulsion of the homologous bacilli. They can be demonstrated in sera that lack any bactericidal action.

REFERENCES

BREKKE, ALEXANDER: Om tyfusvakcinations og seroologiske methoder til bedømmelse av dens effect. (Tillægshefte til Med. Revue, Bergen 1916.)

GAY: Citation by Sormani.

GENGOU: Sur les sensibilisatrices des sérums actifs contre les substances albuminoides. Ann. de l'Inst. Pasteur, 1902, **16**, 734.

GRUBER, M.: Theoretisches über die Antikörper im Blute. Wiener klin. Woch. 1901, **14**, 1244.

LEVADITI: Citation by Sormani.

LIPSTEIN: Die Complementablenkung bei bactericiden Reagenzglasversuchen und ihre Ursache. Centralbl. f. Bakter., Orig., 1902, **31**, 460.

MORESCHI, C.: Zur Lehre von den Antikomplementen. Berl. klin. Woch., 1905, **42**, 1225.

NEISSER, M., AND WECHSBERG, F.: Ueber die Wirkungsart bactericider Sera. Münch. med. Woch., 1901, **48**, 697.

SORMANI, B. P.: Eine neue Erklärung des Neisser- und Wechsbergschen Phänomens vermittels des Phänomens der spezifischen Sprödigkeit. Zeits. f. Immunitätsforsch., Orig., 1916, **24**, 336.

THJØTTA, TH.: On the bacteriology of dysentery in Norway. The Jour. of Bacteriology, 1919, **4**, 355.

THE RELATION OF THE RATE OF ABSORPTION OF ANTIGEN TO THE PRODUCTION OF IMMUNITY

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Studies of the rate of antigen absorption in sensitized and immunized animals, as contrasted with the rate of absorption in normal animals, have shown that previous treatment with an antigen confers upon an animal an increased power of absorption for that antigen. This fact was established with respect to sensitized rabbits and guinea-pigs by Doerr and Pick (1), Friedberger and Lurà (2), Römer and Viereck (3), and others. Smith and Cook (4) confirmed the work of Doerr and Pick and further (5) demonstrated that absorption of antigen in immunized animals proceeds much more rapidly than in sensitized or normal animals.

As it is evident that upon sensitization and particularly upon immunization, an animal acquires increased powers of absorption for the specific antigen, the question arises as to whether the process of immunity production is in any way dependent upon the changed absorptive powers of the organism. In other words, if a high degree of immunity is always accompanied by a marked increase in the rate of absorption of the antigen, can any cause and effect relationship between the two phenomena be established? Or, approaching the subject from another viewpoint, is it possible to demonstrate that conditions which furnish possibilities for increased powers of absorption of antigen also result in the production of a high degree of immunity?

A possible method of investigating this question is suggested by certain facts established by a number of workers with respect to the production of changes in the permeability of the cell membrane through the use of varying concentrations of electrolytes in the surrounding media. Loeb (6), Lillie (7), Osterhout (8), and McClendon (9), have shown that whereas certain marine organisms may be considered to be in equilibrium when the surrounding medium contains a definite proportion of Ca and Na ions, well recognized changes in the cell activities, such as simple stimulation, initiation of cell division, and in extreme cases toxicity and death, may be set up by varying the proportion of these ions. These investigators have demonstrated that the above results are due to changes in the permeability of cell membranes, an increased permeability being caused by an excess of Na ions, a decreased permeability by a slight excess of Ca ions. Clowes (10) in a similar work upon the action of antagonistic electrolytes upon living cells has shown that in higher organisms a disturbance of the equilibrium of the cells can be effectively produced by the use of calcium chloride on the one hand and by sodium citrate upon the other.

With these facts as a basis for further procedure, it seemed possible that by introducing an excess of Na ions or of Ca ions into the blood of experimental animals, changes might be effected in their powers of absorption for an antigen simultaneously injected. To obtain the maximum effect of these electrolytes upon antigen absorption, it seemed advisable to administer them in connection with the usual immunizing procedure, that is in a series of five or more injections. By means of such a procedure a series of observations could be made during the entire course of immunization, and any cumulative effects, as well as any immediate changes due to the action of the electrolytes, could be observed.

In carrying out the experiment, as outlined above, the electrolytes used were calcium chloride and sodium citrate. These were employed in $\frac{M}{10}$ concentration in amounts of 1 cc., toxicity tests upon both electrolytes having shown that this amount usually caused no marked disturbance when given intravenously to rabbits of 1500 to 2000 grams weight. As antigen, a simple protein, egg albumen, was chosen, since the course of absorption of a simple protein can be more readily followed by means of the precipitin reaction than can be accomplished by any of the usual in vitro tests upon a more complex antigen, as for instance a bacterial emulsion.

The rate of absorption of antigen into the blood was measured by bleeding the animals before, and at intervals following each injection and titering these bleedings with an anti-egg precipitating serum for their content in egg albumen. In view of the importance ascribed by various workers (Hektoen (11), Melnikowa and Wersilowa (12)) to the leucocyte reaction in connection with any attempts to change the reactivity of an organism to an antigen, leucocyte counts were made before and at intervals after each injection. The rapidity of antibody formation under the conditions of the experiment was measured by titering the bleedings taken before each injection and a bleeding taken ten to fourteen days after the last injection for precipitins for egg albumen. Further details of the procedure are given in experiment 1.

Experiment 1

Three groups of animals were treated as follows:

Group 1. Rabbits 1, 2, and 3 received for five successive times at five-day intervals intravenous injections of 1 cc. of $\frac{m}{10}$ sodium citrate and immediately following, intraperitoneal injections of 1 cc. of a 2 per cent solution of powdered egg albumen.

Group 2. Rabbits 4, 5, and 6 received for five successive times at five-day intervals intravenous injections of 1 cc. of $\frac{m}{10}$ calcium chloride and immediately following, intraperitoneal injections of 1 cc. of a 2 per cent solution of powdered egg albumen.

Group 3. Rabbits 7, 8, and 9, controls, received at five-day intervals five intraperitoneal injections of 1 cc. of a 2 per cent solution of powdered egg albumen.

Bleedings were taken from all animals immediately preceding each injection and one, two, four, six, twenty-four, and forty-eight hours following. The anti-egg precipitating serum used for the titration of the content of each bleeding in egg albumen was 1: 12800 in titer. The usual precipitin procedure was employed, 0.1 cc. of this serum being added to 2 cc. of dilutions of 1:50, 1:100, 1:200, 1:400, and 1:800 of the serum obtained from the bleedings. In recording the time required for the absorption of antigen, the highest dilution of the serum in which a positive reaction was obtained is given to indicate the relative concentration of albumen in the serum as well as the time interval. These values are given in table 1. In no case was egg albumen present in the bleedings taken immediately preceding the injections.

Leucocyte counts made before each injection and for periods of four to eight hours following are recorded in table 2.

Data in regard to the course of antibody production of each rabbit are given in table 3.

Examination of the data given above shows that there are considerable differences between rabbits receiving electrolytes and the control animals. It is evident that the administration of electrolytes influences very markedly the rate of absorption of antigen, for while antigen was first detected in the blood of the control animals at an interval of four hours after the intraperitoneal injection, the animals receiving sodium citrate gave evidences of absorption in every case after an interval of one hour. Of the animals receiving calcium chloride, on the other hand, no. 5 showed no evidence of absorption at any time, no. 4 gave positive tests for antigen in the blood after an interval of twenty-four hours in four out of five injections, and in no. 6 antigen was absorbed into the blood after an interval of six to eight hours. The most significant feature of these results, however, is the fact that variations in antibody content of these animals were parallel to the differences found in the rates of absorption of antigen. Precipitins appeared more rapidly and in very much higher concentrations in the rabbits of the sodium citrate series than in the control animals. The reverse was true of the animals receiving calcium chloride, one rabbit of this group giving no positive reactions.

With respect to the leucocyte count there was throughout a considerable variation in the individual rabbits. In the control animals there were no marked reactions. In the rabbits receiving sodium citrate there was a rather pronounced fall in the count

TABLE 1

Relative rates of absorption of antigen

ELECTROLYTE	RABBIT		FIRST APPEARANCE OF ANTIGEN IN THE BLOOD AFTER									
			Injection 1		Injection 2		Injection 3		Injection 4		Injection 5	
	Number	Weight	Time	Dilution	Time	Dilution	Time	Dilution	Time	Dilution	Time	Dilution
		grams	*hours*		*hours*		*hours*		*hours*		*hours*	
Sodium citrate........	1	1650	1	1:100	1	1:200	1	1:100	1	1:100	1	1:200
	2	1600	1	1:50	1	1:50	1	1:100	1	1:200	1	1:200
	3	1800	1	1:100	1	1:100	1	1:200	1	1:200	1	1:200
Calcium chloride.....	4	1600	24	1:100	No absorption		24	1:50	24	1:50	24	1:50
	5	2000	No absorption		No absorption		No absorption		No absorption		No absorption	
	6	1500	8	1:50	8	1:50	8	1:50	8	1:100	6	1:100
None, control........	7	2000	5	1:100	6	1:100	4	1:100	4	1:200	4	1:200
	8	1550	4	1:100	4	1:100	4	1:50	4	1:100	4	1:100
	9	1500	4	1:50	4	1:100	4	1:100	4	1:100	4	1:200

TABLE 2

Leucocyte counts

ELECTROLYTE	RABBIT	INJECTION 1		INJECTION 2		INJECTION 3		INJECTION 4		INJECTION 5	
		Time	Count	Time	Count	Time	Count	Time	Count	Time	Count
Sodium citrate...........	1	Before	11,400	Before	10,400	*		Before	9,800		
		1 hr.	3,400	1 hr.	6,100			1 hr.	4,600		
		2 hr.	9,400	4 hr.	6,000			2 hr.	4,600		
		4 hr.	6,500					6 hr.	6,800		
	2	Before	9,000	Before	10,000			Before	9,000		
				1 hr.	4,500			1 hr.	8,400		
				2 hr.	6,000			2 hr.	6,000		
				4 hr.	9,000			3 hr.	10,000		
	3	Before	10,700	Before	9,000	Before	9,800			Before	10,000
		1 hr.	5,600	1 hr.	5,600	1 hr.	7,000			1 hr.	6,800
		3 hr.	6,400	2 hr.	7,000	3 hr.	7,800			3 hr.	8,400
		5 hr.	9,000	4 hr.	9,600	6 hr.	10,000			5 hr.	11,000
Calcium chloride.........	4	Before	9,000	Before	9,600	Before	11,800			Before	15,000
		1 hr.	4,600	1 hr.	20,000	1 hr.	7,500			1 hr.	16,800
		2 hr.	10,000	4 hr.	24,000	3 hr.	21,000			2 hr.	11,000
		4 hr.	9,100	8 hr.	10,000	8 hr.	12,000			5 hr.	16,000
	5	Before	10,000	Before	9,600	Before	12,000	Before	11,500	Before	10,400
		1 hr.	6,000	1 hr.	8,000	1 hr.	10,000	1 hr.	10,000	1 hr.	7,600
		2 hr.	8,000	4 hr.	10,000	3 hr.	11,800	3 hr.	10,800	2 hr.	8,000
		4 hr.	9,800	8 hr.	9,800	4 hr.	11,600	4 hr.	12,000	5 hr.	9,800
	6	Before	13,000	Before	10,800			Before	10,500		
		1 hr.	9,600	1 hr.	9,000			1 hr.	7,700		
		2 hr.	10,400	2 hr.	9,800			2 hr.	10,000		
		6 hr.	9,600	4 hr.	10,600			4 hr.	10,500		

None, control...........	7	Before	11,700	Before	12,200	Before	13,100	Before	11,600	Before	15,300
		1 hr.	11,000	1 hr.	12,200	1 hr.	12,600	1 hr.	9,300	1 hr.	7,800
		2 hr.	17,600	2 hr.	14,200	2 hr.	16,500	2 hr.	9,900	2 hr.	2,400
		5 hr.	12,800	4 hr.	14,600	4 hr.	12,400	4 hr.	10,000	4 hr.	9,400
	8	Before	9,500	Before	8,500	Before	13,900	Before	10,900	Before	13,300
		1 hr.	13,000	1 hr.	9,300	1 hr.	17,100	1 hr.	3,900	1 hr.	23,000
		2 hr.	9,400	2 hr.	9,800	3 hr.	11,400	3 hr.	7,800	3 hr.	17,000
		4 hr.	11,800	4 hr.	8,400	4 hr.	13,500	4 hr.	9,500	4 hr.	14,000
	9	Before	15,800	Before	16,900	Before	15,000	Before	12,000		
		1 hr.	9,600	1 hr.	10,900	1 hr.	15,000	1 hr.	9,800		
		2 hr.	7,500	4 hr.	13,500	2 hr.	10,000	2 hr.	8,400		
		4 hr.	6,500			6 hr.	11,200	4 hr.	11,000		

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* The leucocyte count was not taken because of the occurrence of a severe reaction following the injection of sodium citrate.

one to two hours after injection, and this fall was not followed by a leucocytosis. In the calcium chloride series there was usually a drop in the count the first hour after injection. This was occasionally followed by a leucocytosis with a return to normal in four to five hours. With the exception of the leucopenia observed in the animals receiving sodium citrate, however, the reaction in none of the rabbits was sufficiently distinct to be of any particular significance. The leucocyte reaction cannot, therefore, in this series of experiments be considered as an important factor in determining or reflecting changes in immunity production.

TABLE 3

Precipitin content of bleedings

ELECTROLYTE	RABBIT NUMBER	TIME OF FIRST APPEARANCE	TITER OF BLEEDINGS TAKEN 14 DAYS AFTER THE LAST INJECTION
Sodium citrate...	1	Preceding the 5th injection	Positive in 1:51200
	2	Preceding the 5th injection	Positive in 1:25600
	3	Preceding the 5th injection	Positive in 1:51200
Calcium chloride.	4	14 days after 5th injection	Positive in 1:400
	5	0	Negative in 1:100
	6	10 days after 5th injection	Positive in 1:1600
None, control....	7	10 days after 5th injection	Positive in 1:3200
	8	10 days after 5th injection	Positive in 1:3200
	9	10 days after 5th injection	Positive in 1:1600

As the results of the above series indicate that the production of precipitins is distinctly affected by the administration of sodium citrate and calcium chloride during immunization and that the rate of antibody production follows the rate of antigen absorption it is of interest to ascertain whether the same relations hold true with respect to other antibodies. A similar experiment was therefore carried out, in which a series of animals were immunized to typhoid bacilli and the effect of sodium citrate and calcium chloride was determined upon the production of agglutinins and opsonins, as well as precipitins. Actual determinations of the rate of absorption of the antigen were omitted in this series, as a bacterial antigen when killed before injection cannot be readily detected in the animal body. In this case, it was assumed that the electrolytes would act upon the animal in the same way as when actual determinations were made, as in experiment 1.

Experiment 2

Rabbits 10, 11, 12, 13, 14, and 15 received seven intraperitoneal injections of suspensions of heat-killed typhoid bacilli in physiological saline, one hundred million bacteria being given at each injection. The first three injections were given at five-day intervals. A period of ten days with no injections followed, after which the four remaining injections were given at five-day intervals.

Immediately preceding each intraperitoneal injection of typhoid antigen, rabbits 10 and 11 received 1 cc. $\frac{m}{10}$ sodium citrate intravenously, rabbits 12 and 13 received 1 cc. $\frac{m}{10}$ calcium chloride intravenously, while rabbits 14 and 15 served as controls with no intravenous injections.

Bleedings were taken before the first and fourth injections and seven and seventeen days following the last injection. These bleedings are designated as 1, 2, 3, and 4 respectively. Bleedings 1, 2, and 3 were titered for agglutinins only. With bleeding 4 the titers of agglutinins, opsonins, and precipitins were determined. Table 4 is a comparative study of the development of agglutinins in the six animals. Table 5 gives the concentration of agglutinins, opsonins and precipitins of bleeding 4.

It is evident from table 4 that the production of agglutinins is noticeably influenced by the use of electrolytes during immunization. Animals receiving sodium citrate showed throughout a much higher titer than the controls, while animals receiving calcium chloride were lower in titer than the controls. Table 5 shows, moreover, that agglutinins, opsonins, and precipitins followed parallel courses. The results of this experiment, therefore, confirm those of experiment 1, and while direct evidence cannot be brought that in this case the rate of absorption of the antigen has a direct influence upon the degree of immunity produced, it is nevertheless evident that the use of those agents, which caused such marked changes in the rate of absorption of egg albumen, is followed by changes in antibody production to the typhoid bacillus, which are exactly similar to the changes effected in the production of precipitins for egg albumen.

While the influence of electrolytes upon the reactivity of an organism to an antigen is of considerable interest, the fact which is of chief importance for the present discussion is that a rapid rate of absorption of antigen was followed by an increased production of antibody. The reverse was also true—a much retarded rate of absorption of antigen was accompanied by a marked decrease in the production of antibody. These results, when taken into consideration with the statement made previously—namely, that the condition of immunity is accompanied by increased powers of absorption for the specific antigen—may

TABLE 4

Agglutinin titer of bleedings

ELECTROLYTE	RABBIT	BLEEDINGS			
		1	2	3	4
Sodium citrate	10	0	1:640	1:2560	1:10240
	11	0	1:640	1:2560	1:10240
Calcium chloride	12	0	0	1:160	1:320
	13	0	1:80	1:160	1:320
None, control	14	0	1:160	1:640	1:1280
	15	0	1:80	1:320	1:1280

TABLE 5

Antibody content of bleeding

ELECTROLYTE	RABBIT	AGGLUTININS	OPSONIC INDEX	PRECIPITINS
Sodium citrate	10	1:10240	1.91	1:6400
	11	1:10240	1.80	1:3200
Calcium chloride	12	1:320	0.93	1:100
	13	1:320	0.91	Negative in 1:50
None, control	14	1:1280	1.24	1:1600
	15	1:1280	1.14	1:800

be of significance in throwing some light upon the mechanism of immunity production. Certainly support would seem to be given to the idea that the condition of permeability of the cell, with respect to its absorptive powers for the introduced antigen, is a factor of considerable importance in the production of immunity.

REFERENCES

(1) Doerr, R., and Pick, R.: Centralbl. f. Bakteriol., 1912, **62**, 146.
(2) Friedberger, E., and Lurà, A.: Ztschr. f. Immunitätsforsch u. exper. Therap., 1913, **18**, 272.
(3) Römer, P. H., and Viereck, H.: Ztschr. f. Immunitätsforsch. u. exper. Therap., 1914, **21**, 32.
(4) Smith, G. H., and Cook, M. W.: J. Immunol., 1917, **2**, 421.
(5) Smith, G. H., and Cook, M. W.: J. Immunol., 1917, **2**, 269.
(6) Loeb, J.: J. Biol. Chem., 1917, **32**, 147.
(7) Lillie, R. S.: Am. J. Physiol., 1910–1911, **27**, 289.
(8) Osterhout, W. J. V.: Science, 1917, n.s., **45**, 97.
(9) McClendon, J. F.: Am. J. Physiol., 1911–1912, **20**, 302.
(10) Clowes, G. H. A.: J. Physical Chem., 1916, **20**, 407.
(11) Hektoen, L.: J. Infect. Dis., 1916, **19**, 69 and 737.
(12) Melnikowa F. J. and Wersilowa, M. A.: Centralbl. f. Bakteriol., 1912, **66**, 525.

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STUDIES ON THE MENINGOCOCCIDAL ACTIVITY OF BLOOD

TOITSU MATSUNAMI

From the Government Institute for Infectious Diseases of the Imperial University of Tokyo (Director, Prof. Dr. M. Nagayo)

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A method for measuring the bactericidal activity of blood *in vitro* by employing the many stemmed capillary pipets of Wright, has been recently devised by Heist (1). Heist has shown that this method—which having been in part suggested by Prof. B. F. Lacy, has been termed the Lacy-Heist method—may be used for determining resistance or immunity to pneumococcus infection. By employing the same technic, Dr. Kolmer and I (2) have shown the existence of a relation between the meningococcidal action of the blood of normal animals and the resistance of the animal to infection with virulent meningococci, and also that the high natural immunity or resistance of certain of the lower animals to the meningococcus is to be partly ascribed to a higher meningococcidal activity of their blood, and that the bactericidal blood test as described by Dr. Heist, possesses definite value as a test or measure of bactericidal activity of the blood for meningococci *in vitro*.

The object of the present investigation was to determine, whether or not active immunization with virulent meningococci in rabbits will be accompanied by an increase of the meningococcidal activity of the blood, and also to study the nature of the test for the measuring of antimeningococcal activity of the blood *in vitro*.

EXPERIMENTAL

The experiments were conducted with a single strain of normal meningococcus B[1, 2], the virulence of this strain in mice is shown in the results presented in table 1.

The virulence test was conducted according to the method of Hitchens and Robinson (3) with the exception that active guinea-pig serum and serum-water-dextrose broth were used in preparing the meningococcus emulsion, instead of guinea-pig serum alone.

TABLE 1

*Results of virulence tests at varying periods in mice with meningococcus strain B suspended in serum-water-dextrose-broth**

DOSES†	APRIL 23, 1919		APRIL 26, 1919		MAY 19, 1919		JUNE 4, 1919		JUNE 20, 1919	
	Weight	Result	Weight	Result	Weight	Result	Weight	Result	Weight	Result
mgm.	*gms*		*gms*		*gms*		*gms*		*gms*	
1.0	12	D.24 hrs.	13	D.17 hrs.	10	D.48 hrs.	11	D.17 hrs .	11	D.17 hrs.
0.5	11	D.17 hrs.	12	D.17 hrs.	10	D.17 hrs.	11	S.	11	S.
0.25	11	D.24 hrs.	11	D.17 hrs.	10	D.24 hrs.	11	D.20 hrs.	11	D.17 hrs.
0.125	10	S.	10	D.17 hrs.	10	D.24 hrs.	10	S.	10	D.24 hrs.
0.06	10	S.	10	D.17 hrs.	10	S.	10	S.	9	D.24 hrs.
0.03	10	S.	10	S.	10	S.	10	S.	9	S.
Control	10	S.	10	S.	10	S.	10	S.	9	S.
Control	10	S.	9	S.			10	S.		

* These tests were conducted with strain B transplanted every three days on serum-dextrose-agar.

† Doses in 0.4 cc. of serum-water-dextrose-broth.

D. = died.

S. = survived seven days or longer.

Control = injected 0.4 cc. of serum-water-dextrose-broth.

Bactericidal test. The bactericidal tests were conducted principally after the method described by Heist, a brief account of which is as follows:

Several dilutions of the culture are arranged in sterile tubes and allowed to run by capillary attraction into the many stemmed sterile capillary pipets of Wright, numbered respectively, and measuring about

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[1] I am indebted to Dr. K. Iyehara for conducting the identification test for the type of this strain.

[2] This strain was kindly furnished by Department of Serum Therapy of the Institute.

9 cm. in length and about 1 mm. or less in thickness. The emulsion in the pipets is now removed by touching the tip to moist sterile gauze (which attracts the fluid but leaves a film of microörganisms sticking to the wall of the tube) and each is loaded to the same level with blood, secured by pricking the cleaned skin surface. The pipets are now sealed by dipping the tips in melted paraffin and they are incubated for twenty-four hours, when a smear is made from each pipet and stained for meningococci.

In order to measure the bactericidal activity both of normal and immune rabbit blood, I have employed several modified methods besides the original method of Heist, of which a further description will be given in subsequent tests.

MENINGOCOCCIDAL ACTIVITY OF THE BLOOD OF THE NORMAL RABBIT

That the blood of the normal rabbit possesses high bactericidal activity for normal meningococcus, and that these animals are highly resistant to infection with the same strain injected intraperitoneally in doses of culture, according to body weight, comparable with those given to the mice and guinea-pigs has been reported by Dr. Kolmer and myself (2). In the present investigation with normal strain B, it was found, also, that the blood of the normal rabbit possesses about the same high meningococcidal activity, as shown in table 2.

In conducting the test I have employed suspensions of 10 loops of eighteen hour serum-agar culture of meningococcus in 1 cc. of broth undiluted and in four dilutions prepared with serum-water-dextrose-broth, namely 1:5, 1:25, 1:125 and 1:625.

For the method of cloring the distal end of the pipet, instead of dipping it in melted paraffin, a "peep" flame was used, after the method of sealing a Wright's blood capsule; after the blood has been drawn up, the empty end of the pipet is sealed with a flame and then cooled: The blood is drawn toward this sealed end. The distal end, which is now left emptied of blood, is sealed with a "peep" flame, and the first sealed end is opened by filing, and then incubated.

As is to be seen from table 2 the results of the meningococcidal blood tests with rabbit bloods conducted by the Lacy-Heist method as described indicate that the growth of meningococci in the capillary tubes varies in degree, and also that the blood of the normal rabbit possesses high bactericidal activity for meningococci. I have conducted a series of experiments in search of a method which yields suitable results for the purpose of comparing the bactericidal power of the normal and immune rabbit blood as follows:

TABLE 2

Meningococcidal activity of the blood of normal rabbits and rabbits immune to bacteria other than meningococci

RABBIT	WEIGHT	MENINGOCOCCIDAL TESTS				
		Undiluted[*]	1:5	1:25	1:125	1:625
	grams					
Normal 1	2,710	++[†]	–	–	–	–
Normal 2	2,595	++	++	+	+	–
Normal 3	2,350	++	+	–	–	–
Normal 4	2,240	++	–	–	–	–
Normal 5	2,500	++	++	+	–	–
Normal 6	2,450	++	–	–	–	–
Normal 7	1,780	++	++	+	–	–
Normal 8	1,785	++	+	–	–	–
Normal 9	2,540	++	–	–	–	–
Normal 10	3,050	++	++	–	–	–
Normal 11	3,100	++	–	–	–	–
Normal 12	1,450	++	++	+	–	–
Immune B. typhosus	3,100	++	–	–	–	–
Immune gonococcus	3,000	++	++	+	+	–
Immune B. dysenteriae (Shiga)	2,700	++	+	–	–	–
Immune sheep blood cells	3,050	++	+	–	–	–
Immune sheep blood cells	3,100	++	–	–	–	–
Control[‡]		++	++	++	++	++
Plates[§]		Unc.[¶]	Unc.	Unc. to 5,000	3,000 to 500	1,500 to 100

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[*] Prepared by suspending 10 loops of eighteen hour serum-dextrose-agar culture in 1 cc. serum-water-dextrose-broth.

[†] ++ = heavy growth; + = light growth; – = sterile.

[‡] Serum-water-dextrose-broth substituted for blood.

[§] Plates were prepared by drawing the culture into the capillary tubes employed, expelling the culture, and then washing the cocci, adhering to the inner wall, into Petri dishes with twelve changes of serum-water-dextrose-broth; the number of the colonies gives an idea of the number of cocci adhering to the inner wall of a pipet and subject to the bactericidal activity of the blood.

[¶] Colonies per plate; Unc. = too many colonies for counting.

Experiment 1

In order to measure more accurately the growth of meningococci in capillary tubes after the incubation, three sets of experiments were conducted on the same day, with the same blood and culture; namely, the contents of one set of capillary tubes were plated in Petri dishes and the number of colonies of cocci which grew were counted; also the contents of the second set of tubes was cultured on slants of serum-dextrose-agar to determine whether any cocci survived in the tubes; and, furthermore, the stained smears of the contents of the third set of tubes was examined under the microscope for cocci, as described in the original method.

Table 3 shows the results of these experiments: while the results obtained by examining the stained smear proved quite comparable to the others, for the purpose of obtaining an accurate result of the test, the plate method or slope culture method was found to be preferable.

Experiment 2

In this experiment I have studied the influence of the length of the incubation time upon the meningococcidal action of the blood. In Table 4 are shown the results of the meningococcidal blood test, conducted at various lengths of time of incubation; namely, directly after the test, after thirty minutes, after three hours and after twenty-four hours. It will be seen, by this experiment, that the meningococcidal action of the blood is almost completed within three hours after incubation of the tubes has begun.

Experiment 3

In this experiment I have studied the influence of the number of cocci and the dilution of blood upon the bactericidal action of the blood of rabbits. In order to enumerate the number of cocci for the test, I have employed the bacterial emulsion, instead of using the cocci adhering to the inner wall of the pipet: One volume of this bacterial emulsion was mixed with an equal volume of blood in the pipet as follows:

TABLE 3

Results of meningococcidal blood tests with rabbits as determined by stained smear, slope culture and plate count

RABBIT	WEIGHT	STAINED SMEAR					SLOPE CULTURE					PLATE COUNT				
		Meningococcidal test*					Meningococcidal tests*					Meningococcidal test*				
		Undiluted	1:5	1:25	1:125	1:625	Undiluted	1:5	1:25	1:125	1:625	Undiluted	1:5	1:25	1:125	1:625
	gms.															
Normal 9	2,540	++†	–	–	–	–	++	–	–	–	–	Unc.‡	–	–	–	–
Normal 10	3,050	++	+	–	–	–	++	+	–	–	–	Unc.	60	–	–	–
Normal 11	3,100	++	–	–	–	–	++	–	–	–	–	Unc.	–	–	–	–
Immune B. typhosus	3,100	++	–	–	–	–	++	–	–	–	–	Unc.	–	few	–	–
Immune gonococcus	3,000	++	++	–	–	–	++	++	+	–	–	Unc.	Unc.	–	–	–
Immune B. dysenteriae (Shiga)	2,700	++	+	–	–	–	++	++	–	–	–	Unc.	2,400	–	–	–
Control	·	++	++	++	++	++	++	++	++	++	++	Unc.	Unc.	Unc.	Unc.	Unc.

* Conducted with dense emulsion prepared by suspending 10 loops of eighteen hour serum-water dextrose-agar culture in 1 cc. of serum-water-dextrose-broth.

† + + = heavy growth; + = light growth; – = sterile.

‡ Colonies per plate; Unc. = too many colonies for counting.

In these tests plates were prepared by blowing the contents of the capillary tubes into Petri dishes containing serum water, and by washing the inner wall of the pipet several times with serum-broth, and then pouring dextrose-agar into the dishes.

Each pipet is marked at two levels. After the culture was allowed to run into the pipet by capillary attraction and to reach the first mark, the tip of the tube was withdrawn from the culture tube and introduced into the drop of blood, care being taken to avoid air bubbles between blood and culture; the blood flows up by capillary attraction. When the ascending column of the mixture of blood and culture has reached the second mark, the tube is moved aside and mixed well by keeping the tube constantly rotating with a lateral movement and bringing the column of fluid in the pipet up and down to insure a uniform mixing.

TABLE 4

Results of meningococcidal blood tests with rabbits after various lengths of incubation time

RABBIT	WEIGHT	LENGTH OF INCUBATION TIME	MENINGOCOCCIDAL TESTS[*]			
			Undiluted	1:5	1:25	1:125
	grams					
Normal 8	1,785	At once	Unc.[†]	Unc.	5,400	2,700
		30 minutes	Unc.	5,400	240	0
		3 hours	Unc.	1	0	0
		24 hours	Unc.	0	0	0
Normal 9	2,540	At once	Unc.	Unc.	5,400	2,430
		30 minutes	Unc.	8,100	800	30
		3 hours	Unc.	15	0	0
		24 hours	Unc.	0	0	0
Normal 10	3,050	At once	Unc.	Unc.	6,950	2,700
		30 minutes	Unc.	10,800	780	60
		3 hours	Unc.	12	0	0
		24 hours	Unc.	0	0	0

[*] These tests were conducted with a culture prepared by suspending 10 loops of eighteen hour serum-water-dextrose-agar culture in 1 cc. of broth.

[†] Colonies per plate; Unc. = too many colonies for counting.

By employing this method the number of cocci was enumerated on the one hand, and the blood was diluted one to two on the other hand. It is seen from table 5, in which the result of this experiment is given, that the meningococcidal activity of rabbit's blood is reduced by this method as compared with that obtained by the original method.

It was found in these experiments that this meningococcidal blood test, conducted by mixing equal volumes of blood and culture in capillary tubes, incubating for three hours and then plating for the purpose of counting the surviving cocci, may be used for comparing the meningococcidal activity of normal and immune rabbit's blood.

TABLE 5

Meningococcidal activity of rabbit blood, equal volumes of blood and culture being used

RABBIT	WEIGHT	MENINGOCOCCIDAL TESTS*							
		In slope culture				In plate count			
		Undiluted	1:10	1:100	1:1000	Undiluted	1:10	1:100	1:1000
	grams								
Normal 8	1,785	+++	+++	++	++	Unc.‡	Unc.	8,100	600
Normal 9	2,540	+++	+++	++	+	Unc.	10,800	2,700	360
Normal 10	3,050	+++	+++	+++	+	Unc.	13,500	10,800	800
ImmuneB.typhosus.	3,100	+++	+++	+++	+	Unc.	Unc.	Unc.	5,400
Immune gonococcus	3,000	+++	+++	+++	++	Unc.	Unc.	13,500	5,700
Approximate number of cocci employed for the test		10 million	1 million	100 thousand	10 thousand	10 million	1 million	100 thousand	10 thousand

* These tests were conducted with a culture prepared by suspensing 5 loops of eighteen hour serum-water-dextrose-agar culture in 1 cc. of serum-water-dextrose-broth.

† +++ = heavy growth; ++ = moderate growth; + = light growth.

‡ Number of colonies per plate; Unc. = too many colonies for counting.

MENINGOCOCCIDAL ACTIVITY OF THE IMMUNE RABBIT BLOOD FOR MENINGOCOCCI

1. *Method of immunization employed by the author*

A number of rabbits were immunized with meningococci principally after the method of Amoss and Wollstein (4), which was devised for preparation of antimeningococcic serum in the horse. It consisted in inoculating, alternately, living and autolyzed products of meningococci into the vein. Living cultures of meningococci grown on serum-dextrose-agar slant for eighteen hours were given to each rabbit in doses as follows: on the first day 0.02 cc., on the second 0.05 cc. and on the third 0.1 cc. of one loop of cocci suspended in 2 cc. of normal salt solution. After the lapse of seven days a series of three injections of autolyzed meningococci, consisting of the filtrate of a suspension of meningococci in normal salt solution, which had been incubated for twenty-four hours at 37°C., in doses of 2 cc. containing autolysate of 0.1 cc. of one loop of meningococcus culture in 2 cc. of normal salt solution was injected. After the lapse of seven days, for the second time, two series of three injections each of living and autolyzed cocci were given in the same manner. This process of immunization was repeated for the third and the fourth time.

2. The bactericidal blood test

The bactericidal blood test was conducted by the method described above. Equal volumes of blood and culture were mixed in a capillary tube, incubated for three hours, and plated for counting the surviving cocci.

3. The agglutination test

The agglutination test with the serum was conducted before the beginning of each immunization.

The results of a number of these tests are summarized in table 6.

As shown in table 6 the meningococcidal activity of immunized rabbit blood has generally been found to be stronger than that of normal blood or of that drawn before immunization. This increase of bactericidal activity was marked in the blood of rabbits that had received only the first series of the injections of cocci, and distinctly more with rabbits that had received the injections for the second time. But, as may be seen from the results recorded in table 6, the blood of rabbits which had received more than two series of injections have been found to be quite irregular as far as bactericidal activity *in vitro,* is concerned. The bactericidal activity of the blood does not accompany the increase of the agglutination titer of the serum. The bactericidal activity of the blood of rabbits 3 and 7 as regards the tubes which contained the smaller number of cocci, and of

TABLE 6

Results of meningococcidal blood and agglutination tests with immune rabbits for meningococcus strain B

RABBIT NO.	BLOOD		WEIGHT	AGGLUTINATION TITER†	MENINGOCOCCIDAL TESTS*			
					Undiluted	1:10	1:100	1:1000
			grams					
1	Before immunization		2,710	1 : 20	Unc.‡	Unc.	3,780	180
			2,710	1 : 20	Unc.	16,200	4,700	240
			2,710	1 : 20	Unc.	13,500	3,780	300
	After immunization	I	2,500	1 : 40	13,500	2,700	120	12
		II	2,400	1 : 640	5,400	120	120	0
		III	2,450	1 : 640	18,900	3,780	600	8
2	Before immunization		2,595	1 : 20	Unc.	13,500	5,400	300
			2,595	1 : 20	Unc.	Unc.	3,780	420
	After immunization	I	2,450	1 : 40	16,200	5,400	180	10
		II	2,550	1 : 640	3,240	360	60	0
		III	2,500	1 : 640	21,600	3,280	800	12
3	Before immunization		2,350	1 : 10	Unc.	8,100	5,400	360
	After immunization	I	2,250	1 : 80	13,500	4,700	240	180
		II	2,170	1 : 640	8,100	10,800	80	60
		III	2,400	1 :1280	140	0	10,800	5,400
4	Before immunization		2,240	1: 20	Unc.	13,500	7,800	300
	After immunization	I	2,100	1 : 80	10,800	3,200	180	8
		II	1,750	1 : 320	16	5,400	240	0
		III	Died					
5	Before immunization		2,500	1 : 10	Unc.	7,560	8,100	320
	After immunization	I	2,350	1 : 160	8,100	5,400	360	730
		II	Died					
6	Before immunization		2,450	1 : 10	Unc.	Unc.	8,100	600
	After immunization	I	2,300	1 : 80	Unc.	3,780	360	0
		II	2,150	1 : 320	5,400	600	180	320
		III	2,400	1 : 640	8,100	0	120	600

TABLE 6—*Concluded*

RABBIT NO.	BLOOD	WEIGHT	AGGLUTINATION TITER†	MENINGOCOCCIDAL TESTS*			
				Undiluted	1:10	1:100	1:1000
		grams					
7	Before immunization	1,750	1 : 10	27,000‡	10,800	5,400	360
	After immunization I	1,750	1 : 80	18,900	3,780	120	60
	After immunization II	1,950	1 : 640	3,780	600	680	11
	After immunization III	2,000	1 : 640	600	8	5,400	3,240
	After immunization IV	2,100	1 : 1280	18,900	180	800	360
Approximate number of cocci employed for the test........................				2,000,000 to 10,000,000	200,000 to 1,000,000	20,000 to 100,000	2000 to 10,000

* Conducted with an equal volume of blood and culture, prepared by suspending 5 loops of eighteen hour serum-water-dextrose-agar culture in 1 cc. of broth.

† Agglutination tests were conducted at 55° C. for twenty hours.

‡ Colonies per plate; Unc. = too many colonies for counting. Each immunization consisted of three successive injections of living and, after seven days interval, autolyzed product of meningococcus respectively.

rabbits 1 and 2 as regards nearly all the tubes employed in these tests, has been found to be much less after the third series of injections than that of blood taken after the first or second series. In regard to this phenomenon I have found a similar result in a horse highly immunized with meningococci. As shown in table 7 the bactericidal activity of this horse conducted by the method described, has been found surprisingly low compared with that of normal horse blood.

TABLE 7

Showing that highly immunized horse blood is less bactericidal for meningococci than normal horse blood

HORSE	AGGLUTINATION TITER*	MENINGOCOCCIDAL TESTS†
Immune 34....................................	1 : 1280	Uncountable ‡
Normal...	1 : 160	600

* Agglutination tests were conducted at 55° C. for twenty hours.

† Conducted with equal volumes of blood and culture, prepared by suspending 10 loops of eighteen hour serum-dextrose agar culture in 1 cc. of serum-water-dextrose-broth.

‡ Colonies per plate; Uncountable = too many colonies for counting.

This phenomenon may possibly fall in a class with phenomena of a somewhat similar nature. For instance, in the case of agglutination, clumping of the bacteria may not occur in low dilutions of an immune serum, while it may be complete in high dilutions. As another instance, Neufeld (5) and others state that too much agglutinin in a serum inhibits opsonic activity, resulting in irregular and low bactericidal activity for meningococci. It may be that in the process of immunization the blood acquires the power of inhibiting autolysis of meningococci. Whether one or the other of these factors, or a summation of them all is responsible for the facts observed, or whether some other factor, (or factors) is concerned is not determined by the present investigation.

The results of my tests with normal and immune rabbit blood may be summarized as follows:

1. The blood of normal rabbits and rabbits immunized against *B. dysenteriae* (Shiga), *B. typhosus* and gonococci has been found to possess marked bactericidal activity for a virulent normal strain of meningococcus within three hours *in vitro*.
2. The blood of a rabbit immunized against meningococcus by intravenous injection of a living and autolyzed culture of meningococcus has been found to be distinctly more bactericidal than that of normal rabbits or rabbits immunized against *B. typhosus*, *B. dysenteriae* (Shiga) and *gonococcus*.
3. The increase of the meningococcidal activity of the blood of rabbits after immunization has been found to be parallel up to a certain limit with the process of immunization, but further immunization did not appear to show increase of bactericidal activity *in vitro*.

STUDIES OF THE NATURE OF MENINGOCOCCIDAL ACTIVITY OF RABBIT BLOOD IN VITRO

Discovery of the bactericidal activity of the blood for various microorganisms (6) (7) and that of the phenomenon of phagocytosis have thrown a light on the explanation of the resistance of the organism to certain microorganisms. By further investigations it has been proved that the bactericidal activity of the blood is to be ascribed to the action of the various immune bodies or antibacterial substances contained in blood serum or plasma, in phagocytes and in blood platelets. In this respect I have conducted a series of experiments to study the nature of the meningococcidal activity of the blood *in vitro* as follows:

1. *Bactericidal activity of the serum for meningococci*

Potent antimeningococcus serum is generally regarded as possessing a certain meningococcidal activity in addition to specific opsonin, toxin neutralizing antibodies, agglutinins, precipitins and the complement fixing antibodies, and upon these its curative powers probably depend. But the meningococcidal activity of antimeningococcus serum does not appear to be ascribable to the presence of specific bacteriolysin requiring the presence of complement for its lytic activity: While Davis (8) and McKennzy and Martin (9) were able to demonstrate *in vitro* the complemental bacteriolysis with the serum of meningitis patients, Flexner (10) proved that heated serum also possesses bactericidal activity, and he advanced the hypothesis that it is only necessary that the fresh or heated serum should injure the cocci in order that their intracellular enzymes should be rendered active, and thus destroy the microorganism. Jobling (11) and others deny the action of complemental bacteriolysis with the antimeningococcus serum. According to the results obtained by Drs. Kolmer, Toyama and myself (12) (13) while studying the influence of active normal serum (complement) upon meningococci, the bactericidal activity *in vitro* of horse antimeningococcus sera is quite low, although some bactericidal activity is generally apparent as compared with the control, and largely independent of complemental bacteriolysis; it was also found by our experiments, that the higher dilutions of serum not infrequently are more bactericidal than the lower. Jochmann (14) also found this to be the case. In the present investigation I have conducted a comparative study of the meningococcidal activity of rabbit blood and serum and controls, the results of which are given in tables 8 and 9. As shown in table 8 normal rabbit's sera have been found to posses a certain meningococcidal activity, quite comparable with that of the sera of immune rabbits. While the normal rabbit blood is more bactericidal than serum, the meningococcidal activity of the immune rabbit blood has been found to be distinctly stronger than that of

TABLE 8

*Showing that whole blood is more bactericidal for meningococci than serum**

RABBIT	AGGLUTINATION TITER	RESULTS WITH BLOOD AND CULTURE	RESULTS WITH SERUM AND CULTURE
Normal 8	1 : 10	8,100†	18,900
Normal 9	1 : 20	13,500	Unc.
Normal 10	1 : 20	10,800	16,200
Normal 11	1 : 20	13,500	Unc.
Immune 1	1 : 640	600	16,200
Immune 2	1 : 640	60	Unc.
Immune 3	1 : 80	600	16,200
Immune 6	1 : 320	300	8,100
Immune 7	1 : 1280	600	Unc.

* These tests were conducted each with an equal volume of blood or serum with twenty-four hour egg-yolk-dextrose-broth culture; one volume of this culture contained approximately 50,000 meningococci.

† Colonies per plate; Unc. = too many colonies for counting.

TABLE 9

Antimeningococcidal action of normal salt solution, sodium citrate in normal salt solution, cerebrospinal fluid and normal rabbit serum

APPROXIMATE NUMBER OF COCCI EXPOSED TO GERMICIDAL ACTUAL	MENINGOCOCCIDAL TESTS*				
	Normal salt solution	1.5 per cent sodium citrate solution in normal salt solution	Cerebro-spinal fluid	Normal rabbit serum (inactivated at 55°C for thirty minutes	Control (egg yolk dextrose broth)
80,000	0†	0	Uncount.‡	18,900	Uncount.§

* These tests were conducted with meningococci adhering to the inner wall of a capillary tube; culture of which is prepared by suspending 5 loops of eighteen hour serum-dextrose agar culture in 1 cc. of broth.

† Number of colonies per plate: Uncount.‡ = Colonies in these plates appeared to be comparable to the plate containing the cocci exposed to germicidal action of fluids; Uncount.§ = Colonies in these plates appeared to be more than in the plate containing the cocci exposed to germicidal action of fluids.

immune serum. The results of the experiment indicate, therefore, that the bactericidal activity of the rabbit blood can not be ascribed totally to the serum. As found by Flexner (10) and others, normal salt solution and 1.5 per cent sodium citrate in normal salt solution were very toxic for meningococci, while the cerebrospinal fluid did not appear to be toxic for them within three hours *in vitro*, as shown in table 9.

2. Bactericidal activity of the defibrinated blood for meningococci

The meningococcidal activity of the defibrinated rabbit's blood, prepared by shaking with glass beads, has been found to be quite similar to that of the serum and to lack the high bactericidal activity of the whole blood as shown in table 10.

TABLE 10

*Showing that whole blood is more bactericidal for meningococci than defibrinated blood**

RABBIT	AGGLUTINATION TITER	RESULT WITH WHOLE BLOOD AND CULTURE	RESULT WITH DEFIBRINATED BLOOD AND CULTURE
Immune 1	1 : 640	600†	18,900
Immune 2	1 : 640	120	Unc.
Immune 6	1 : 320	360	16,200
Immune 7	1 : 1280	600	Unc.

* In these tests a twenty-four hour egg-yolk-dextrose-broth culture was employed; the number of meningococci in one volume of culture being approximately 50,000.

Agglutination tests were conducted at 55° C. or twenty hours.

† Number of colonies per plate; Unc. = too many colonies for counting.

3. Bactericidal activity of rabbit's blood, consisting of blood cells and serum

The bactericidal test with the blood, which is prepared by a method shown in table 11, consisting of whole formed constituents of the blood and serum, has been conducted with the meningococci. The result of this experiment, shown in table 11, indicates that the meningococcidal activity of the untreated blood is still distinctly higher than that of the blood cells plus serum, although the bactericidal activity of the latter has been found to be generally slightly stronger than that of serum alone or of defibrinated blood. It will be seen by this experiment that the difference in bactericidal activity between the two kinds of blood is not to be ascribed to the difference in the number of leucocytes acting as phagocytes; as the leucocytes contained in both samples of blood may be regarded as about the same in number.

TABLE 11

*Showing that whole blood is more bactericidal for meningococci than blood cells plus serum**

RABBIT	AGGLUTINATION TITER	RESULT WITH WHOLE BLOOD AND CULTURE	RESULT WITH BLOOD CELLS, SERUM AND CULTURE
Immune 1	1 : 640	300†	10,800
Immune 2	1 : 640	600	16,200
Immune 6	1 : 320	300	8,100
Immune 7	1 : 1280	60	18,900

* These tests were conducted with a twenty-four hour egg-yolk-dextrose-broth culture in equal volume and incubated for three hours.

Number of cocci in one volume of the culture being approximately 50,000.

† Number of colonies per plate.

The blood used for this experiment, consisting of blood cells and serum, was prepared as follows:

One cubic centimeter of immune rabbit blood was taken from an ear vein by puncture into a syringe containing 4 cc. of sterile 1.5 per cent solution of sodium citrate in normal salt solution. This blood-citrate mixture was transferred to a sterile centrifuge tube and centrifuged. The supernatant fluid was transferred into a second sterile centrifuge tube and centrifuged again at a high speed. The supernatant fluid was then drawn off. The blood cells left in the first and second centrifuge tubes were mixed with the active serum, which was previously separated by coagulation of 1 cc. of the same rabbit blood, thus making the mixture of blood cells and serum correspond to a certain amount of blood. This mixture of blood cells and serum was then used for comparative bactericidal blood tests for meningococci with whole blood.

4. *Bactericidal activity of citrated blood*

For a study of the bactericidal activity of the blood prevented from coagulating I have conducted the test with blood, the coagulation of which was prevented by the use of sodium citrate. The result is given in table 12. Since sodium citrate in normal salt solution is very toxic for meningococci, as shown in table 9, and inasmuch as Otani (25) found that spontaneous phagocytosis in a mixture of citrated blood of certain microorganisms occur quite freely the results of experiments with citrated blood, may, strictly speaking, not be comparable with those with untreated blood, but the bactericidal activity of both bloods being concerned, the untreated whole blood was found more bactericidal for the meningococcus than blood which was prevented from coagulating by the use of sodium citrate.

TABLE 12

*Showing that whole blood is more bactericidal for meningococci than citrated blood**

RABBIT	AGGLUTINATION TITER	RESULT WITH WHOLE BLOOD AND CULTURE	RESULT WITH CITRATED BLOOD AND CULTURE
Immune 2	1 : 1280	5400†	Uncount.
Immune 7	1 : 1280	8100	Uncount.

* These tests were conducted with a twenty-four hour egg-yolk-dextrose-broth culture in equal volume and incubated for three hours.

Number of cocci in one volume of the culture counting approximately 80,000.

† Number of cocci per plate; Uncount. = too many colonies for counting.

Citrated blood was prepared by adding 0.02 gram of sodium citrate to 1 cc. of blood.

5. Influence of coagulation of the blood upon the meningococcidal activity of the blood

Since the results of experiment 1, 2, 3 and 4 indicate that the bactericidal activity of whole blood is greater than that of serum alone or defibrinated blood or blood cells plus serum or citrated blood, the reason for the difference of the bactericidal activity *in vitro* between whole blood and others appears to be in the process of coagulation of the blood. Although the direct influence of coagulation of blood upon meningococci may be negligible for the comparative study of the bactericidal blood test *in vitro* by reason of the fact, that the meningococci will not be destroyed by the process of coagulation of blood alone if the blood does not possess bactericidal activity as indicated in the results of experiments with mice and other animals (2), coagulation taking place in the test in both immune and normal blood. It is, however, highly probable, that the indirect influence of the process of coagulation plays a considerable and important rôle in the bactericidal blood test *in vitro* with respect to the meningococci. As pointed out by Wright and Dr. Heist (1), when the blood is allowed to clot in the capillary tube a "semi-solid" is formed, and in this condition and furthermore by the possible influence of chemical changes of blood which occur in the clotting, phagocytosis, by which meningococci undergo intracellular digestion, may be more active than that of the blood in a fluid state. Therefore the influence of coagulation of blood, at least as one factor of difference between the whole blood and defibrinated blood and blood cells plus serum or citrated blood may not be a negligible one with respect to the bactericidal activity for the meningococcus *in vitro*. But it appears difficult to ascribe this difference solely to the influence of coagulation of the blood, because the degree of that influence upon the meningococcidal activity of blood can not be determined, inasmuch as a method to measure the bactericidal activity of whole blood which can be prevented from coagulating without making any change in the original nature of blood, is unknown. According to Dr. Heist (1), the blood of pigeon, which is highly resistant against pneumococcus infection, has distinct antipneumococcic factors *in vitro*, while this activity is not to be found in serum or in defibrinated blood or in blood of the pigeon influenced by coagulation.

The results of the preceding study on the significance of bactericidal activity of the blood for meningococci may be summarized as follows:

1. The meningococcidal activity of whole blood of normal rabbit *in vitro* was found to be stronger than that of serum.
2. The meningococcidal activity of serum and of defibrinated blood of the immune rabbit for meningococci was found comparable with that of the normal rabbit and slightly less strong than that of blood cells plus serum of the immune rabbit.
3. It was found that the meningococcidal activity of whole blood of the immune rabbit is strikingly stronger than that of serum, defibrinated blood, blood cells plus serum or citrated blood of the same rabbit.

4. It is suggested, that at least one factor explaining the difference in meningococcidal activity *in vitro* of whole blood, defibrinated blood, citrated blood and serum is because coagulation in the bactericidal blood test with whole blood, favors phagocytosis of the meningococci.

SUMMARY

1. It has been found by the pipet method that normal rabbit blood and serum are capable of killing considerable numbers of virulent normal meningococci *in vitro* within three hours.

2. The meningococcidal activity *in vitro* of normal rabbit blood was found to be increased up to a certain limit by the intravenous injection of the living and autolyzed meningococci. After that, further immunization did not appear to increase bactericidal activity, was generally rather irregular and not infrequently even decreased meningococcidal activity of the blood. The more highly immunized rabbit's blood was found sometimes less bactericidal than that of slightly immunized rabbit's blood.

3. The meningococcidal activity of normal rabbit's serum has been found not to be increased by artificial immunization and also to be comparable with that of defibrinated blood of an immune rabbit.

4. The meningococcidal activity *in vitro* of immune rabbit's blood was found by the pipet method to be distinctly stronger than that of the serum, of defibrinated blood or of blood consisting of blood cells and serum or of citrated blood. It was suspected that at least one factor in explaining the higher meningococcidal activity *in vitro* of immune rabbit's blood compared with defibrinated blood, citrated blood and serum lies in the influence of coagulation of the blood, which is permitted in the regular blood test as described, favoring the phagocytosis of meningococci.

5. The meningococcidal blood test can not be accepted on the basis of the present investigation for the purpose of measuring or determining the artificially induced immunity against meningococci.

However, as pointed out by Dr. Heist (1) the bactericidal blood test described possesses the advantage of employing whole blood; hence when this method is used any mechanism of immunity existing in the blood, may be brought into direct relation with the microorganism. Moreover, in this test any antibacterial factors existing in the blood, and the influence of individual variation of fluid constituent as well as phagocytes in blood, upon the bactericidal activity of the blood, have equal chance to come into play. Furthermore, it was found that a parallelism between the bactericidal activity of the blood and resistance to certain bacteria including meningococci, exists under normal conditions (1) (2). Therefore, the bactericidal blood test described may be regarded as a method possessing definite value for the measuring the natural resistance of the organism for certain microorganisms especially for meningococci, inasmuch as with respect to meningococci no accurate method sufficiently reliable to serve as a definite measure of antibody content has yet been devised for measuring immunity to this microorganism (16).

I wish to express my thanks to Dr. Yutaka Nakamura for advice and asistance in carrying out this work.

REFERENCES

(1) Heist, G. D., Solis-Cohen, S., and Solis-Cohen, M.: The bactericidal action of whole blood, with a new technique for its determination. Jour. of Immunology, 1918, **3**, 261.

(2) Matsunami, T., and Kolmer, J. A.: The relation of the meningococcidal activity of the blood to resistance to virulent meningococci. Jour. of Immunology, 1918, **3**, 201.

(3) Hitchens, A. P., and Robinson, G. H.: Standardization of antimeningitis serum. Jour. of Immunology, 1916, **2**, 345.

(4) Amoss, H., and Wollstein, M.: A method for the rapid preparation of antimeningitis serum. Jour. Exper. Med., 1916, **23**, 403.

(5) Neufeld, F.: Bakteriotropine und Opsonine. Kolle und Wassermann's Handbuch d. Patholog. Mikro., 1913, **2**, I, 416.

(6) Friedberger, E.: Die Bakteriziden Sera. Jolle und Wassermann's Handbuch d. Patholog. Mikro., 1913, Bd. **2**, I, 298.

(7) Zinsser, H.: Infection and Resistance. McMillian, 1918, Ed. II, 134.

(8) Davis, D. J.: Studies in meningococcus infections. Jour. Infect. Diseases, 1905, **2**, 602–619.

(9) McKenzie, D., and Martin, W. B. M.: Serum therapy in cerebrospinal meningitis. Jour. Path. and Bact., 1908, **12**, 539–549.

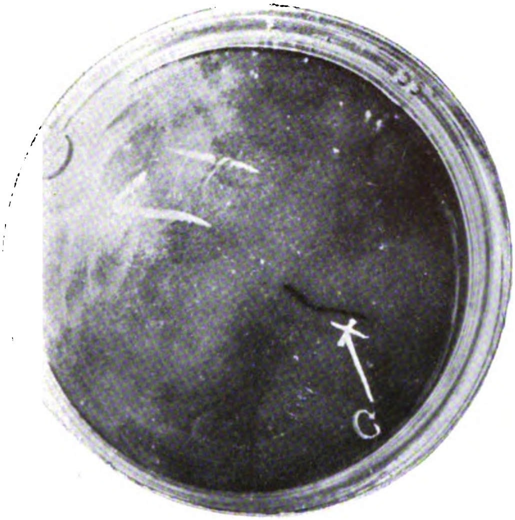
(10) Flexner, S.: Contribution to the biology of diplococcus intracellularis. Jour. Exper. Med., 1907, **9**, 105–141.
(11) Jobling, J. W.: Standardization of the antimeningitis serum. Jour. Exper. Med., 1909, **11**, 614–621.
(12) Kolmer, J. A., Toyama, I., and Matsunami, T.: The influence of active normal serum (complement) upon meningococci. I. Jour. of Immunology, 1918, **3**, 157.
(13) Matsunami, T., and Kolmer, J. A.: The influence of active normal serum (complement) upon meningococci. II. Jour. of Immunology, 1918, **3**, 177.
(14) Jochmann, G.: Versuche zur Serodiagnostik und Serumtherapie der epidemischen Genickstarre. Deutsch. Med. Wochenschr., 1906, **32**, 788–793.
(15) Otani, M.: On the acceleration of phagocytosis in the citrated blood. The Kitasato Archives of Experim. Med., 1918, **2**, 2, 147.
(16) Kolmer, J. A.: Infection, Immunity and Specific Therapy. Saunders, 1917, Ed. II, 792.

DESCRIPTION OF FIGURES

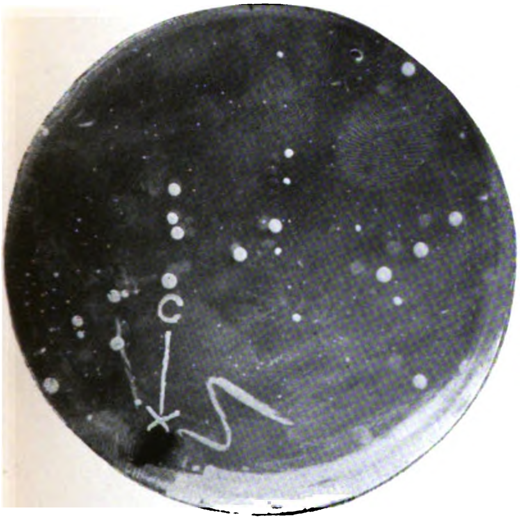
Showing colonies of meningococci grown in Petri dishes, survived of the meningococcidal blood test in the capillary tube; C = Coagula of blood. (The author's modified method for estimating the result of the meningococcidal blood test in the capillary tube.)

I. Plate of strong meningococcidal blood
II. Plate of marked meningococcidal blood
III. Plate of weak meningococcidal blood

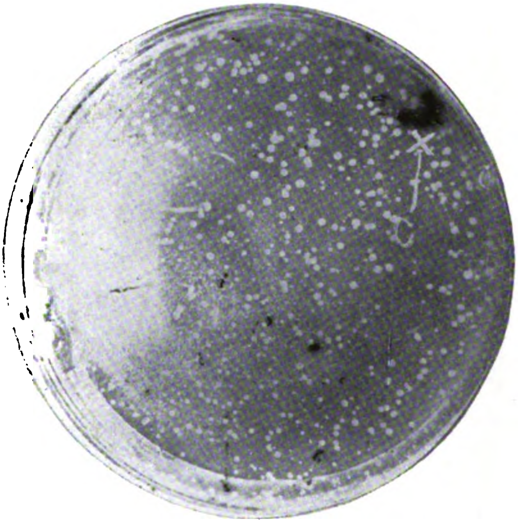
I



II



III



NATURAL ANTIHUMAN HEMOLYSINS AND HEMAGGLUTININS IN HORSE SERA IN RELATION TO SERUM THERAPY

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Inasmuch as the intravenous injection of persons with large amounts of immune horse serum as in the serum treatment of pneumonia, is sometimes followed by symptoms not referable to anaphylaxis, the purpose of this investigation was to study normal and immune horse sera for hemagglutinins and hemolysins for human erythrocytes to ascertain whether intravascular agglutination or hemolysis are responsible in part for these symptoms.

This study was conducted with agglutination and hemolysin tests *in vitro* in which normal and immune horse sera and the erythrocytes of different persons were employed; horse sera were found to contain relatively large amounts of agglutinins for the erythrocytes of some rabbits and were injected intravenously into these animals to determine the effects of agglutination and hemolysis *in vivo*.

Williams and Patterson (1) tested 19 horse serums and found 12 to contain agglutinins for human erythrocytes, and suggest that in fatalities following large injections of serum, the possibility of agglutination of red corpuscles is to be considered; they also suggest the advisability of testing the sera of horses against human corpuscles and of rejecting any horse whose serum had distinct agglutinating power.

In this investigation 30 horse sera have been used, 28 being obtained from immunized and 2 from normal horses; all sera were kindly furnished by Dr. John Reichel of the Mulford Biological Laboratories. The majority of immune sera contained a preservative as prepared for distribution and administration to persons.

RESULTS OF AGGLUTINATION TESTS

Macroscopical tests. Macroscopical agglutination tests were conducted by mixing in small test tubes 0.1 cc. of each horse serum unheated with 1 cc. of 1 per cent suspension of washed erythrocytes from 9 to 12 different persons, the final dilution being about 1:11; results based upon tests with the erythrocytes of any one person were found only approximately correct since the agglutinins in horse sera for human erythrocytes occur in groups, similar to the groups of agglutinins and hemolysins in human sera for the erythrocytes of the lower animals (2).

TABLE 1

Agglutinins in unheated horse sera for human erythrocytes

HORSE SERUM UNHEATED 5.1 CC.	HUMAN ERYTHROCYTES, 1 CC. OF 1 PER CENT SUSPENSION								
	1	2	3	4	5	6	7	8	9
Antistreptococcus..	−[*]	−	−	−	−	−	−	−	−
Antistreptococcus..	−	−	−	−	−	−	−	−	−
Antistreptococcus..	−	−	−	−	−	−	−	−	−
Antistreptococcus..	−	−	−	−	−	−	−	−	+
Antistreptococcus..	−	−	−	−	−	−	−	+	−
Normal............	−	−	−	−	−	−	−	−	−
Normal............	−	−	−	−	−	−	−	−	−
Antipneumococcus..	+	+	−	+	−	+	+	−	−
Antistreptococcus..	−	−	−	+	−	−	−	−	−
Antipneumococcus..	−	−	−	−	−	−	+	−	−
Antistreptococcus..	−	−	+	−	−	−	−	−	−
Antistreptococcus..	+	+	+	+	+	+	+	+	+

[*] Incubation in water bath at 38°C. for one hour. Readings made after standing overnight in refrigerator; −, no agglutination; +, = agglutination.

The mixtures and the controls were incubated in a water-bath for one hour at 38°C. and the results were read after the mixture had stood in a refrigerator over night.

The results observed with 12 sera tested with the erythrocytes of 9 different persons, are given in table 1; the results with 28 sera tested with the corpuscles of each of 12 different persons are shown in table 2.

As indicated in these tables about 50 per cent of horse sera showed the presence of hemagglutinins with this technic in a final dilution of about 1:11, for the erythrocytes of certain persons; no single serum contained hemagglutinins for the erythrocytes of all persons tested, indicating the presence of group hemagglutinins in varying amounts for the different groups of human erythrocytes.

TABLE 2

Agglutinins in unheated horse sera for human erythrocytes

UNHEATED HORSE SERUM 0.1 cc.	HUMAN ERYTHROCYTES 1 cc. OF 1 PER CENT SUSPENSION											
	1	2	3	4	5	6	7	8	9	10	11	12
Antistreptococcus.......	–[*]	–	–	–	–	–	–	–	+	–	–	–
Antistreptococcus.......	–	–	–	–	–	–	–	–	–	–	–	–
Antistreptococcus.......	–	–	–	–	–	–	–	–	–	–	–	–
Antistreptococcus.......	–	–	–	–	–	–	–	–	–	–	0	0
Antistreptococcus.......	–	–	–	–	–	–	–	–	–	–	–	–
Normal.................	–	–	–	–	–	–	–	–	–	–	–	–
Normal.................	–	–	–	–	–	–	–	–	–	–	–	–
Antipneumococcus......	–	–	–	–	–	–	–	–	+	–	–	–
Antistreptococcus.......	–	–	–	–	–	–	–	–	–	–	–	–
Antipneumococcus......	–	–	–	–	–	–	–	–	–	–	–	–
Antistreptococcus.......	–	–	–	–	–	–	–	–	–	–	–	–
Antistreptococcus.......	–	–	–	–	–	–	–	–	+	–	–	–
Antipneumococcus......	–	–	–	–	–	–	–	–	–	–	–	–
Antipneumococcus......	–	–	–	–	–	–	–	–	+	–	–	+
Antipneumococcus......	–	–	–	–	–	+	–	–	–	–	–	–
Antipneumococcus......	–	–	–	–	–	+	–	–	+	–	–	+
Antipneumococcus......	–	–	–	–	–	–	–	–	–	–	–	–
Anti-influenzal..........	–	–	–	–	–	–	–	–	+	–	–	+
Anti-influenzal..........	–	–	–	–	–	–	–	–	–	+	–	–
Anti-influenzal..........	–	–	–	–	–	+	–	–	–	–	–	–
Anti-influenzal..........	–	–	–	–	+	–	–	–	–	–	–	–
Anti-influenzal..........	–	–	–	–	–	–	–	–	–	–	–	–
Antimeningococcus......	–	–	–	–	+	–	–	–	–	–	–	–
Antimeningococcus......	–	–	–	–	–	–	+	–	–	–	–	–
Antistreptococcus.......	–	–	–	–	–	–	–	–	–	–	–	–
Antistreptococcus.......	–	–	–	–	–	–	–	–	–	–	–	–
Antistreptococcus.......	–	–	–	–	–	–	–	–	–	–	–	–
Antistreptococcus.......	–	–	–	–	–	–	–	–	–	–	–	–

[*] –, No agglutination; +, agglutination.

Microscopical tests, however, in which lower dilutions of sera were employed, showed the presence of these hemagglutinins in a larger percentage of sera.

Microscopical tests. Microscopical tests were conducted by mixing on cover-glasses one loopful of serum and one loopful of a 1 per cent suspension of washed cells, the results being read ten to fifteen minutes later; the final dilutions with this technic were 1:2 and showed the presence of small amounts of hemagglutinins in practically all horse sera examined.

TABLE 3

Microscopical agglutination tests with horse sera and human erythrocytes

HORSE SERUM	HUMAN ERYTHROCYTES											
	1	2	3	4	5	6	7	8	9	10	11	12
Normal	+*	+	−	+	−	+	+	+	±	0	+	±
Normal	+	+	+	+	+	+	−	±	±	0	±	+
Antipneumococcus	−	−	−	+	−	±	+	−	±	0	+	+
Antistreptococcus	−	−	+	+	±	+	+	−	+	0	+	+
Antipneumococcus	+	+	+	+	+	+	−	−	+	+	+	+
Antistreptococcus	+	+	+	+	+	−	+	+	+	+	+	+
Antistreptococcus	−	−	+	+	+	+	+	+	−	−	+	−
Antipneumococcus	−	±	±	+	±	−	−	−	−	0	+	−
Antipneumococcus	±	±	+	+	−	−	+	+	±	±	+	−
Antipneumococcus	+	+	+	+	+	+	+	+	+	−	+	+
Antipneumococcus	+	+	+	+	+	+	+	+	+	+	+	+
Antipneumococcus	±	+	+	±	−	−	+	±	±	−	+	+
Anti-influenzal	+	+	+	+	−	+	+	−	+	−	±	±
Anti-influenzal	+	+	+	+	±	+	+	±	±	±	+	±
Anti-influenzal	−	−	+	+	+	+	+	±	±	0	+	−
Anti-influenzal	±	±	+	+	+	+	+	−	+	0	−	±
Antimeningococcus	−	−	−	+	±	+	+	−	+	0	+	±
Antimeningococcus	+	±	±	±	±	+	+	±	±	+	±	±
Antitetanus	+	+	+	+	+	+	+	+	+	+	+	+
Antitetanus	−	−	+	+	+	+	+	±	+	0	+	+
Antistreptococcus	±	−	±	−	−	−	+	−	±	−	±	−
Antistreptocoecus	−	+	±	+	+	+	+	±	+	0	+	−
Antistreptococcus	−	±	±	+	−	±	+	±	+	0	+	±
Antistreptococcus	+	+	±	+	+	+	+	±	+	0	+	±

* −, No agglutination; ±, partial agglutination; +, strong agglutination.

The results of one series of such tests with 24 sera and the erythrocytes of each of 12 persons, are shown in table 3; the results observed with 3 sera and the corpuscles of 24 persons are given in table 4.

As shown in these tables all normal and immune horse sera contain hemagglutinins for human erythrocytes when tested microscopically with a technic similar to that proposed for the detection of human isohemagglutinins; when tested with the corpuscles of a number of different persons, however, only a few sera show the presence of hemagglutinins for the cells of all persons, so that a single serum may not agglutinate the corpuscle of a certain individual.

TABLE 4

Microscopical agglutination test with horse sera and human erythrocytes

HORSE SERUM	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
Normal I...	−	±	±	±	−	−	−	+	±	±	−	−	+3	±	−	−	−	−	−	−	−	−	±	−
Normal II..	−	+3	+3	+3	+2	+2	+2	+3	+3	±	+2	+	+2	±	+2	−	+2	+3	±	±	±	−	+3	+2
Normal III..	+	+2	+3	+2	±	+3	+3	+	±	±	−	−	+	+2	+2	−	+2	−	±	±	±	−	+	±

−, Negative no agglutination; ±, very doubtful agglutination; +, very weak agglutination; +2, marked positive agglutination; +3, very strong agglutination.

TABLE 5

Quantitative agglutination tests with preserved horse sera and human erythrocytes

SERA	CORPUSCLES I		CORPUSCLES II		CORPUSCLES III		CORPUSCLES IV	
	Macroscopic	Microscopic	Macroscopic	Microscopic	Macroscopic	Microscopic	Macroscopic	Microscopic
Antistreptococcus........	1:4	1:4	1:8	1:8	1:32	1:8	1:16	1:2
Antipneumococcus.......	1:8	1:4	1:16	1:8	1:16	1:8	1:16	1:8
Antipneumococcus.......	1:2	1:4	1:4	1:2	1:2	1:2	1:2	1:2
Antitetanus..............	1:2	None	None	None	1:4	None	1:2	None
Anti-influenzal..........	1:16	1:8	1:16	1:8	1:16	1:8	1:32	1:8
Anti-influenzal..........	1:8	1:8	1:16	1:8	1:16	1:8	1:8	1:4
Normal..................	1:16	1:4	1:16	1:8	0	0	0	0
Normal..................	1:32	1:8	1:64	1:16	0	0	0	0
Normal..................	1:16	1:4	1:64	1:32	0	0	0	0

The *amount* of hemagglutinins in *preserved* horse sera for human erythrocytes is relatively small; when tested macroscopically in final dilution of 1:11 only about 50 per cent of sera contain hemagglutinins for the corpuscles of some persons (tables 1 and 2); in higher dilutions fewer and fewer sera are found to contain hemagglutinins and only about 20 per cent were found to yield positive macroscopical reactions in dilutions higher than 1:20 and none with dilutions higher than 1:64. A few examples of quantitative macroscopical and microscopical tests are shown in table 5; contrary to expectations the macroscopical tests frequently gave higher readings than the microscopical tests, the differences being largely ascribed to the time allowed for agglutination, being but fifteen minutes for the latter and many hours for the macroscopical tests. As shown later fresh horse sera contain larger amounts of agglutinins for human cells, but the results shown in table 5 indicate the range of agglutination with sera several weeks or months of age and preserved with tricresol at fluctuating temperatures.

RESULTS OF HEMOLYSIN TESTS

Tests for natural antihuman hemolysins in normal and immune horse sera were conducted by mixing, in small test tubes, 0.1 cc. serum, 1 cc. of 1 per cent suspension of washed corpuscles and 1 cc. of a 1:20 dilution of the hemolysin-free sera of several guinea-pigs for complement; incubation was conducted in a water bath at 38°C. for one hour and the results read the following day.

The results observed with 12 unheated sera tested with the erythrocytes of 9 persons are shown in table 6; inasmuch as the anticomplementary activity of these sera may have inhibited hemolysis, additional tests were conducted with 27 sera previously heated at 56°C. for one-half hour, with the corpuscles of 12 persons; the results are given in table 7.

As shown in these tables ordinary normal and immune horse sera prepared for administration to persons, are practically free of hemolysin for human erythrocytes.

Fresh horse sera, however, may contain small amounts of natural antihuman hemolysins as shown in table 8; this table shows the results of macroscopical agglutination tests with 3 fresh normal horse sera in final dilution of 1:11 with the corpuscles of 24 different persons and hemolysin tests conducted as

TABLE 6

Results of hemolysin tests with unheated horse sera and human erythrocytes

HORSE SERUM UNHEATED 0.1 cc.	HUMAN ERYTHROCYTES, 1 cc. OF 1 PER CENT SUSPENSION								
	1	2	3	4	5	6	7	8	9
Antistreptococcus	N.H.*	N.H.	N.H.	N.H.	N.H.	N.H.	N.H.	N.H.	N.H.
Antistreptococcus	N.H.	N.H.	N.H.	N.H.	N.H.	N.H.	N.H.	N.H.	N.H.
Antistreptococcus	N.H.	N.H.	N.H.	N.H.	N.H.	N.H.	N.H.	N.H.	N.H.
Antistreptococcus	N.H.	N.H.	N.H.	N.H.	N.H.	N.H.	N.H.	N.H.	N.H.
Antistreptococcus	N.H.	N.H.	N.H.	N.H.	N.H.	N.H.	N.H.	N.H.	N.H.
Normal	N.H.	N.H.	N.H.	N.H.	N.H.	N.H.	N.H.	N.H.	N.H.
Normal	N.H.	N.H.	N.H.	N.H.	N.H.	N.H.	N.H.	N.H.	N.H.
Antipneumococcus	N.H.	N.H.	N.H.	N.H.	N.H.	N.H.	N.H.	N.H.	N.H.
Antistreptococcus	N.H.	N.H.	N.H.	N.H.	N.H.	N.H.	N.H.	N.H.	N.H.
Antipneumococcus	N.H.	N.H.	N.H.	N.H.	N.H.	N.H.	N.H.	N.H.	N.H.
Antistreptococcus	N.H.	N.H.	N.H.	N.H.	N.H.	N.H.	N.H.	N.H.	N.H.
Antistreptococcus	N.H.	N.H.	N.H.	N.H.	N.H.	N.H.	N.H.	N.H.	N.H.

* Complement furnished by mixed guinea-pig sera in dose of 1 cc. of 1:20 dilution (= 0.05 cc. undiluted serum). N.H. = no hemolysis.

TABLE 7

Results of hemolysin tests with heated horse sera and human erythrocytes

INACTIVATED HORSE SERA 0.1 cc.	HUMAN ERYTHROCYTES											
	1	2	3	4	5	6	7	8	9	10	11	12
Antistreptococcus	N	N	N	N	N	N	N	N	N	N	N	N
Antistreptococcus	N	N	N	N	N	N	N	N	N	N	N	N
Normal	N	N	N	N	N	N	N	N	N	N	N	N
Normal	N	N	N	N	N	N	N	N	N	N	N	N
Antipneumococcus	N	N	N	N	N	N	N	N	N	N	N	N
Antistreptococcus	N	N	N	N	N	N	N	N	N	N	N	N
Antipneumococcus	N	N	N	N	N	N	N	N	N	N	N	N
Antistreptococcus	N	N	N	N	N	N	N	N	N	N	N	N
Antistreptococcus	N	N	N	N	N	N	N	N	N	N	N	N
Antipneumococcus	N	N	N	N	N	N	N	N	N	N	N	N
Antipneumococcus	N	N	N	N	N	N	N	N	N	N	N	N
Antipneumococcus	N	N	N	N	N	N	N	N	N	N	N	N
Antipneumococcus	N	N	N	N	N	N	N	N	N	N	N	N
Antipneumococcus	N	N	N	N	N	N	N	N	N	N	N	N
Anti-influenzal	N	N	N	N	N	N	N	N	N	N	N	N
Anti-influenzal	N	N	C	N	N	N	N	N	N	N	N	N
Anti-influenzal	N	N	N	N	N	N	N	N	N	N	N	N
Anti-influenzal	N	N	N	N	N	N	N	N	N	N	N	N
Anti-influenzal	N	N	N	N	N	N	N	N	N	N	N	N
Antimeningococcus	N	N	N	N	N	N	N	N	N	N	N	N
Antimeningococcus	N	N	N	N	N	N	N	N	N	N	N	N
Antitetanus	N	N	N	N	N	N	N	N	N	N	N	N
Antitetanus	N	N	N	N	N	N	N	N	N	N	N	N
Antistreptococcus	N	N	N	N	N	N	N	N	N	N	N	N
Antistreptococcus	N	N	N	N	N	N	N	N	N	N	N	N
Antistreptococcus	N	N	N	N	N	N	N	N	N	N	N	N
Antistreptococcus	N	N	N	N	N	N	N	N	N	N	N	N

N, No hemolysis; C, hemolysis.

described above, complement being furnished in constant dose of 1 cc. of a 1:20 dilution of the hemolysin-free sera of several guinea-pigs.

As shown in table 8, a fresh serum may contain hemolysin for the corpuscles of a certain individual but not for all persons; this indicates that the natural antihuman hemolysins in horse serum exist in groups similar to the group of hemagglutinins and analagous to the groups of natural hemolysins and hemagglutinins in human sera for the corpuscles of the lower animals described by Kolmer, Trist and Flick (2).

TABLE 8

Results of hemolysin and hemagglutination tests with fresh horse sera and human erythrocytes

HORSE SERA	HUMAN RED BLOOD CELLS																							
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
Fresh normal...	—[*] S	— N	— N	— N	— N	— N	— N	— N	— N	— N	— N	— N	— N	— N	— N	— N	— N	— N	— N	— N	— N	— N	— N	— N
Fresh normal...	— N	— N	— N	+ N	— N	+ N	± N	— N	— N	— N	— N	— N	— N	— N	— N	— N	— N	— N	— N	— N	— N	— N	— N	— N
Fresh normal...	— S	— N	— N	— N	— N	— N	— N	— N	— N	— N	— N	— N	— N	— N	— N	— N	— N	— S	— N	— S	— N	— S	— S	— S

[*] —, No agglutination; +, agglutination; S, slight hemolysis; N, no hemolysis.

These experiments have also shown that in a macroscopical test hemolysin may be present and hemagglutinins absent or as is more frequently observed, hemagglutinins may be present and hemolysins absent.

NATURE OF HEMAGGLUTININS AND HEMOLYSINS IN HORSE SERA FOR ERYTHROCYTES

Natural hemolysins and hemagglutinins are divided into two groups according to resistance to the influence of heating sera at 56°C. for thirty minutes; those that are destroyed or masked by this exposure are designated as thermolabile in contradistinction to those that are more resistant or thermostabile. The majority of natural hemolysins and hemagglutinins in human sera for human erythrocytes and the erythrocytes of the lower animals have been found to be thermolabile (2); heating sera apparently destroys some of these antibodies outright, while others become masked (3). In general terms thermolabile hemolysins and hemagglutinins are also quite susceptible to the deteriorating influence of age, desiccation and strong chemical germicides and antiseptics.

These factors have an important bearing upon the present subject, inasmuch as horse immune sera prepared for administration to persons are generally weeks or months old, preserved with various chemical germicides notably tricresol and phenol, and are frequently kept at ordinary room temperature for varying periods of time, all of which may bring about deterioration of any natural hemagglutinins and hemolysins for human erythrocytes present in the fresh serum.

TABLE 9

The influence of desiccation upon hemagglutinins in three horse sera for the erythrocytes of four rabbits

CONDITION OF SERA	SERUM I				SERUM II				SERUM III			
	Cells 1	Cells 2	Cells 3	Cells 4	Cells 1	Cells 2	Cells 3	Cells 4	Cells 1	Cells 2	Cells 3	Cells 4
Fresh	+*	+	+	+	+	+	+	+	+	+	+	+
Dried 5 days	±	+	±	±	+	±	±	+	±	−	±	−
Dried 12 days	±	−	−	±	±	−	−	±	+	−	−	−

*+, agglutination; ±, doubtful agglutination; −, no agglutination.

Experiments have shown that the hemagglutinins and hemolysins in horse sera for human erythrocytes are thermolabile and thermostabile; the hemolysins are even more susceptible to heat than the hemagglutinins and are almost entirely thermolabile.

Of interest in this connection is the influence of desiccation upon the natural hemagglutinins in horse sera; Karsner and Koeckert (4) and Kolmer (5) have shown that drying human sera upon cover glasses may result in deterioration of isohemagglutinins and we have found the same true of the agglutinins in horse sera for human and rabbit corpuscles. In table 9 are shown the results of tests conducted with rabbit erythrocytes; some of the hemagglutinins deteriorated more rapidly than others. The tests were conducted with cover glasses bearing one loopful of serum dried at room temperature; the corpuscles were used in 1 per cent suspensions, a loopful being rubbed up in the dried serum on each cover glass and examined microscopically fifteen minutes later.

Additional experiments were conducted with rabbit corpuscles and the fresh sera of three normal horses, portions of which were kept in a refrigerator at 0° to 2°C. with and without tricresol (0.25 per cent), at room temperature (about 20°C.) and in an incubator (38°C.). Preliminary microscopical agglutination tests showed that these sera contain agglutinins for a mixed antigen of erythrocytes from several rabbits in final dilutions as high as 1:80 to 1:100, and the influence of temperature and 0.25 per cent tricresol upon natural hemagglutinins in horse serum, was studied with tests in which mixed suspensions of cells from two or more rabbits were employed.

The results of these experiments summarized in tables 10, 11 and 12 have shown that tricresol itself in 0.25 per cent had no influence upon the hemagglutinins, neither aiding in their destruction nor materially protecting against deterioration; temperature, however, had a marked influence inasmuch as sera kept in the incubator and at room temperature showed marked deterioration of hemagglutinins at the end of one week (titers reduced to about 1:20), still more at the end of two weeks (titers about 1:8) and almost complete destruction at the end of three months (titers about 1:2). Sera kept in a refrigerator at 0° to 2°C., however, showed much less deterioration, the titers being at the end of three months reduced from about 1:80 to 1:32; there was little or no difference between sera kept at this temperature with and without 0.25 per cent tricresol.

These experiments have shown, therefore, that natural hemagglutinins and hemolysins in horse sera are quite susceptible to deterioration and when immune horse sera preserved with tri-

TABLE 10

The deterioration of agglutinins for rabbit erythrocytes in normal horse sera kept at 39°C.

SERA KEPT FOR	SERUM I		SERUM II		SERUM III	
	No preservative	Tricresol	No preservative	Tricresol	No preservative	Tricresol
One week	1:20	1:20	H*	1:20	H	1:20
Two weeks	H	1:8	H	1:12	H	1:8
Fourteen weeks	H	1:2	H	1:4	H	None

*H, Hemolysis due to bacterial contamination.

TABLE 11

The deterioration of agglutinins for rabbit erythrocytes in normal horse sera kept at room temperature

SERA KEPT FOR	SERUM I		SERUM II		SERUM III	
	No preservative	Tricresol	No preservative	Tricresol	No preservative	Tricresol
One week	1:40	1:40	1:20	1:20	H*	1:20
Two weeks	None	1:20	H	1:16	H	1:10
Fourteen weeks	None	1:4	H	1:8	H	1:8

*H, hemolysis due to bacterial contamination.

TABLE 12

The deterioration of agglutinins for rabbit erythrocytes in normal horse sera kept at 0 to 2°C.

SERA KEPT FOR	SERUM I		SERUM II		SERUM III	
	No preservative	Tricresol	No preservative	Tricresol	No preservative	Tricresol
One week	1:80	1:40	1:60	1:40	1:100	1:60
Two weeks	1:40	1:80	1:80	1:80	1:60	1:80
Fourteen weeks	1:16	1:32	1:80	1:22	1:60	1:32

cresol are kept at room temperature or even in an ordinary refrigerator (8° to 16°C.), as they are likely to be under commercial conditions, these substances may be expected to deteriorate and their clinical significance lessened thereby.

THE INTRAVENOUS INJECTION OF RABBITS WITH HORSE SERA CONTAINING HEMAGGLUTININS AND HEMOLYSINS

As previously stated fresh horse sera generally contain agglutinins and hemolysins for rabbit erythrocytes; tables 13 and 14 give the results of macroscopical tests with three fresh normal horse sera and the cells of four different rabbits. In the hemolysin tests complement was furnished by 1 cc. of 1:20 hemolysin from guinea-pig sera.

TABLE 13

Agglutinins in normal unheated horse sera for rabbit erythrocytes (macroscopical tests)

SERA	HIGHEST POSITIVE DILUTIONS			
	Rabbit 1	Rabbit 2	Rabbit 3	Rabbit 4
Horse 1	1:40	1:20	Less than 1:20	1:20
Horse 2	1:40	1:40	Less than 1:20	1:40
Horse 3..[4]	1:40	1:20	1:20	1:80

TABLE 14

Hemolysins in normal unheated horse sera for rabbit erytrhocytes

SERA	HEMOLYSIS			
	Rabbit 1	Rabbit 2	Rabbit 3	Rabbit 4
Horse 1	S.H.*	S.H.	N.H.	M.H.
Horse 2	S.H.	M.H.	S.H.	C.H.
Horse 3	M.H.	S.H.	S.H.	C.H.

C.H., Complete hemolysis; M.H., marked hemolysis; S.H., slight hemolysis; N.H., no hemolysis.

Rabbits injected intravenously with normal horse sera containing agglutinins for their cells in macroscopical tests in dilutions as high as 1:40, also hemolysins, showed no ill effects; 10 cc. of serum per kilogram of weight and even as much as 20 cc. per kilogram injected intravenously rather rapidly (about 5 cc. per minute) produced no discernible immediate or late reactions, as respiratory disturbances, hematuria and hemoglobinuria.

Inasmuch as commercial immune horse sera contain comparatively lesser amounts of agglutinins for human erythrocytes and practically no hemolysins at all it is very doubtful if their administration to persons even in large amounts intravenously, is followed by sufficient agglutination and hemolysis *in vivo* to produce discernible symptoms directly referable to these phenomena.

CONCLUSIONS

1. Practically all horse sera contain agglutinins for human erythrocytes; these hemagglutinins occur in groups.
2. The amounts of agglutinins for human erythrocytes in horse sera are highest in fresh sera; in ordinary horse immune sera preserved with tricresol at fluctuating temperatures, the amounts of hemagglutinins are relative small, about 50 per cent of such sera containing agglutinins in dilution 1:2 to 1:11 and only about 20 per cent agglutinating 1:20 or higher.
3. Fresh horse sera may contain natural antihuman hemolysins, but these are found only occasionally in older and preserved sera.
4. Agglutinins in horse sera for human erythrocytes are thermolabile and thermostabile; the hemolysins are almost entirely of the thermolabile variety.
5. Hemagglutinins in normal and immune horse sera deteriorate rapidly and to a large extent in sera kept at body and ordinary room temperatures; when kept at a low temperature deterioration is slower and less marked. Desiccation tends to destroy these natural hemagglutinins.
6. Horse sera contain larger amounts of agglutinins and hemolysins for rabbit than for human erythrocytes; these occur in groups.
7. The intravenous injection of large amounts of horse sera containing agglutinins and hemolysins for rabbit erythroctyes into rabbits, produced no ill effects and no symptoms referable to intravascular agglutination or hemolysis.
8. It is highly probably that the intravenous injection of preserved horse serum does not introduce sufficient agglutinin and hemolysin for human erythrocytes to produce ill effects referable to intravascular agglutination and hemolysis; this is particularly true if the serum is diluted and slowly injected.

REFERENCES

(1) Williams, H. U., and Patterson, H. A.: The agglutination of human red blood corpuscles by horse serum. Jour. Amer. Med. Assoc., 1918, 70, 1754–1755.

(2) Kolmer, J. A., Trist, M. E., and Flick, A. M.: A study of the natural thermolabile and thermostabile hemolysins and hemagglutinins in human sera in relation to the Wassermann reaction. Amer. Jour. Syphilis, 1920, 4, 111-135.

(3) Kolmer, J. A.: The nature of thermolabile hemolysins. Jour. Immunology (in press).

(4) Karsner, H. T., and Koeckert, H. L.: Jour. Amer. Med. Assoc., 1919, 73, 1207-1210.

(5) Kolmer, J. A.: The influence of desiccation on human normal isohemagglutinins. Jour. Amer. Med. Assoc., 1919, 73, 1459–1460.

AN ATTEMPT TO PRODUCE SPECIFIC IMMUNE AGGLUTININS AND HEMOLYSINS FOR THE FOUR GROUPS OF HUMAN ERYTHROCYTES

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Antibodies occurring naturally or normally in sera are seldom if ever as resistant to deleterious influences as immune antibodies, those produced by the body cells during active immunization; accordingly, the natural agglutinins and hemolysins in human sera for the erythrocytes of persons and of the lower animals are as a group thermolabile or heat sensitive and likewise highly susceptible to the deterioration of age and desiccation (1). Some of the natural hemolysins and hemagglutinins and particularly those for sheep corpuscles are more resistant than others, but the isoagglutinins and isohemolysins, which are of particular interest in relation to blood transfusion and the grouping of bloods, are quite sensitive. As a general rule the natural hemolysins are more susceptible to destruction by heat, age and desiccation than the agglutinins and in human serum containing an agglutinin and hemolysin for a particular group of corpuscles, the hemolysin disappears before the agglutinin.

Karsner and Koeckert (2) have reported deterioration of isoagglutinins in sera dried on cover glasses after Sanford's method and have made the interesting observation that group specificity is lost in dried sera kept for several weeks; we have noted with this method considerable reduction in the isoagglutinins in sera dried on cover glasses which may amount to a total loss with some sera and particularly during the first few days after drying. Isoagglutininins persisting in the dried sera over the first one to four days were found to keep fairly well over a period of several weeks when preserved according to Sanford's directions; accordingly only picked sera, that is, those containing more isoagglutinins than the average serum, should be chosen for drying on cover glasses and these should be tested a few days later to determine whether or not the agglutinins have escaped destruction. Probably a better practice is to add a small amount of preservative to a serum (0.2 per cent tricresol) and keep it at a low temperature, frozen if possible, to preserve the isoagglutinins at least, if not the isohemolysins as well.

PURPOSE OF INVESTIGATION

Inasmuch as it has been shown that immunization of the lower animals with type I pneumococci results in the production of antibodies largely or entirely specific for the type and that immunization with one of the types of meningococci also results in the production of a highly monovalent serum, we have considered the possibility of producing agglutinins and hemolysins specific for each of the four groups of human erythrocytes (Moss classification) by immunizing rabbits with group I, II, III and IV corpuscles; we had no reasons for hoping for the production of absolutely specific sera and expected the production of smaller amounts of group agglutinins and hemolysins but surmised that these may be removed by absorption, the specific antibodies alone remaining in diminished amounts but yet in greater degree than found in human sera and more resistant to deleterious influences, as are immune antibodies generally. The purpose of this investigation was to put this proposition to trial; the technic employed and the results observed are briefly stated.

TECHNIC

Classification of erythrocytes. Human erythrocytes were secured from specimens of blood submitted for the Wassermann test, the sera being first removed and a small amount of saline added to the coagula with gentle breaking up and the release of corpuscles. With this method the erythrocytes of twenty or more persons were grouped at one time and not only furnished an abundance of corpuscles satisfactory for immunizing purposes, but sera as well for further groupings.

The groupings were made after Sanford's modification of the Moss classification (3) by a microscopic reaction in which group II and group III sera are employed; the tests were conducted on cover glasses by mixing two loopfuls of group II serum with one loopful of corpuscles suspension and repeating with group III serum on a second cover glass; these preparations and a corpuscle control in which saline solution replaced serum, were suspended in hanging drop slides and examined after standing thirty minutes at room temperature (this period has yielded us better results than a ten or fifteen minute period for agglutination); the reactions were read after the convenient diagram published by Sanford, which may be stated as follows:

Group II serum + and group III serum + = group I corpuscles.
Group II serum − and group III serum × = group II corpuscles.
Group II serum × and group III serum − = group III corpuscles.
Group II serum − and group III serum − = group IV corpuscles.

Immunization of rabbits. Rabbits were immunized either with the corpuscles of one individual or with a mixture of the erythrocytes of several persons belonging to the same group; the broken up coagula were filtered through cotton to remove fibrin and coagula and the corpuscles washed three times with saline solution. After the last washing the corpuscles of the four groups were kept in formalized saline solution (4) in a refrigerator and used as required over a period of several weeks.

Rabbits were immunized with corpuscles of the four groups by injecting intravenously 0.5 cc. of washed packed cells suspended in 5 cc. of sterile saline solution every five days.

Agglutination tests. At intervals during the immunization each rabbit was bled four days after the previous injection and its serum was tested for agglutinins and hemolysins for groups I, II, III and IV corpuscles; in conducting these tests a *macroscopic* technic was employed as follows:

The serum was inactivated at 56°C. for thirty minutes and four series of dilutions were made in small test tubes ranging from 1:10 upward in amounts of 1 cc.; to each tube of series I including a control was added 1 cc. of a one per cent suspension of group I corpuscles; to series II was added group II corpuscles and so on with groups III and IV corpuscles. The total volume in each tube of the four series was 2 cc.; after one hour incubation in a water bath at 38°C. the tubes were placed in a refrigerator over night and the readings were made the following day with the naked eye. In the tables are given the highest dilutions of serum producing definite agglutination; as a general rule the readings were quite easy and sharp although microscopic tests may have carried the titers somewhat higher.

Hemolysin tests. These were conducted in exactly the same manner except that complement was added to each tube excepting the corpuscle controls, in the form of 0.1 cc. of 1:1½ dilutions of the fresh mixed sera of guinea-pigs. *In the tables are given the smallest amounts of undiluted serum producing complete hemolysis.*

RESULTS

The results are summarized in tables 1, 2, 3 and 4.

The results of preliminary experiments conducted with group I and group II corpuscles were encouraging and lead us to believe that it may be possible to produce specific agglutinins and hemolysins for the corpuscles of the four groups (5); the results of the first preliminary experiment are shown in table 1 in which the serum of rabbit 1 immunized with group I corpuscles agglutinated group I corpuscles in dilution of 1:1280 and groups II, III and IV in 1:320, after six injections of corpuscles; the results of the second preliminary experiment are shown in table 2 in which rabbit 1 immunized with the corpuscles of a group II person agglutinated group II corpuscles in final dilution of 1:640 and groups I, III and IV corpuscles in dilutions of 1:80, after four injections of cells. Why these two animals reacted as they did is unknown because a larger series of immunizations conducted with equal care has not shown similar results, as may be seen by glancing over the tables.

As a general rule the serum of each rabbit contained just a little more agglutinin for the erythrocytes of the group used in immunization than for the erythrocytes of the other groups; in several instances, however, these differences in agglutinin content were not apparent.

It is more difficult properly to interpret the results of the hemolysin tests because of variation in resistance of the corpuscles to hemolysis; as a general rule, however, it may be stated that the serum of a given rabbit was equally hemolytic for the corpuscles of all four groups, although instances are shown in the tables when the titer was somewhat higher for the corpuscles of the homologous group, that is, for the corpuscles of the group employed in immunization.

Absorption of the sera removed all agglutinins and hemolysins equally; even with sera containing agglutinins 1:1280 for the corpuscles of a certain group and 1:640 for the corpuscles of the other group, we have not been able to devise a method of absorption that did not remove all or almost all agglutinins and hemolysins at the same time. These absorption tests were conducted by adding to each cubic centimeter of the heated serum of a rabbit immunized with group I corpuscles, 0.5 cc. of the washed packed cells of each of groups II, III and IV at the same time, mixing and placing in a refrigerator over night. On the next day the mixtures were placed in a water bath at 38°C. for one hour and centrifuged; usually the sera required one or more additional absorptions at 38°C. for one hour before microscopic tests with undiluted serum showed the absence of all agglutinins for groups II, III and IV corpuscles; unfortunately, no agglutinins for group I corpuscles would be found or were present in but traces. In absorbing the sera of rabbits immunized with group II corpuscles, the corpuscles of groups I, III and IV were used and so on with the sera of rabbits immunized with the corpuscles of groups III and IV; the results were similar and we have not so far been successful in differentiating them by processes of absorption, even when one is present in larger amount.

TABLE 1

The results of hemagglutination and hemolysin tests conducted with corpuscles of groups I, II, III and IV and the sera of rabbits immunized with group I corpuscles

RABBITS	IMMUNIZATION	GROUP I CORPUSCLES		GROUP II CORPUSCLES		GROUP III CORPUSCLES		GROUP IV CORPUSCLES	
		Agglutinins	Hemolysins	Agglutinins	Hemolysins	Agglutinins	Hemolysins	Agglutinins	Hemolysins
No. 1	After 5 injections	1:640	0.025	1:320	0.025	1:320	0.025	1:320	0.025
	After 6 injections	1:1280	0.012	1:320	0.025	1:320	0.025	1:320	0.025
No. 2	After 4 injections	1:640	0.05	1:320	0.05	1:320	0.05	1:160	0.05
No. 3	After 4 injections	1:640	0.012	1:320	0.025	1:320	0.025	1:320	0.012
	After 5 injections	1:12680	0.006	1:1000	0.012	1:1000	0.006	1:640	0.006

TABLE 2

The results of hemagglutination and hemolysin tests conducted with corpuscles of groups I, II, III and IV and the sera of rabbits immunized with group II corpuscles

RABBITS	IMMUNIZATION	GROUP I CORPUSCLES		GROUP II CORPUSCLES		GROUP III CORPUSCLES		GROUP IV CORPUSCLES	
		Agglutinins	Hemolysins	Agglutinins	Hemolysins	Agglutinins	Hemolysins	Agglutinins	Hemolysins
No. 1	After 4 injections	1:80	0.0015	1:640	0.025	1:80	0.0015	1:80	0.0015
No. 2	After 4 injections	1:160	0.0015	1:320	0.0015	1:320	0.0015	1:160	0.0015
	After 5 injections	1:640	0.0125	1:1280	0.0015	1:640	0.006	1:640	0.003
No. 3	After 3 injections	1:640	0.05	1:1280	0.025	1:640	0.05	1:640	0.05
	After 5 injections	1:640	0.006	1:1280	0.003	1:640	0.025	1:640	0.025
No. 4	After 3 injections	1:640	trace	1:1280	trace	1:640	trace	1:1280	trace
No. 5	After 3 injections	1:640	0.025	1:1280	0.006	1:640	0.0125	1:320	0.006
No. 6	After 5 injections	1:320	trace	1:320	trace	1:160	none	1:320	none
No. 7	After 5 injections	1:320	0.0125	1:1280	0.0125	1:320	0.0125	1:320	0.0125
No. 8	After 8 injections	1:640	0.025	1:640	0.025	1:320	0.025	1:640	0.025
No. 9	After 8 injections	1:320	0.006	1:320	0.006	1:320	0.006	1:320	0.006
No. 10	After 4 injections	1:1280	0.0125	1:1280	0.006	1:1280	0.006	1:640	0.006
No. 11	After 8 injections	1:320	0.025	1:320	0.025	1:320	0.006	1:160	0.0125
	After 9 injections	1:1280	0.025	1:640	0.025	1:640	0.006	1:320	0.006

TABLE 3

The results of hemagglutination and hemolysin tests conducted with corpuscles of groups I, II, III and IV and the sera of rabbits immunized with group III corpuscles

RABBITS	IMMUNIZATION	GROUP I CORPUSCLES		GROUP II CORPUSCLES		GROUP III CORPUSCLES		GROUP IV CORPUSCLES	
		Agglutinins	Hemolysins	Agglutinins	Hemolysins	Agglutinins	Hemolysins	Agglutinins	Hemolysins
No. 1	After 3 injections	1:640	trace	1:640	trace	1:640	0.025	1:320	0.025
No. 2	After 3 injections	1:1280	0.025	1:1280	0.025	1:1280	trace	1:1280	0.025
No. 3	After 3 injections	1:80	none	1:80	none	1:160	none	1:80	none
No. 4	After 5 injections	1:1280	0.006	1:1280	0.006	1:1280	0.006	1:640	0.006
No. 5	After 4 injections	1:320	0.025	1:320	0.025	1:320	0.0125	1:640	0.025
No. 6	After 4 injections	1:640	0.003	1:640	0.0125	1. 640	0.0015	1:640	0.0125

TABLE 4

The results of hemagglutination and hemolysin tests conducted with corpuscles of groups I, II, III and IV and the sera of rabbits immunized with group IV corpuscles

RABBITS	IMMUNIZATION	GROUP I CORPUSCLES		GROUP II CORPUSCLES		GROUP III CORPUSCLES		GROUP IV CORPUSCLES	
		Agglutinins	Hemolysins	Agglutinins	Hemolysins	Agglutinins	Hemolysins	Agglutinins	Hemolysins
No. 1	After 3 injections	1:640	trace	1:640	trace	1:320	trace	1:640	0.05
	After 5 injections	1:640	0.05	1:640	0.05	1:640	0.05	1:640	0.05
	After 7 injections	1:640	0.025	1:640	0.05	1:1280	0.025	1:1280	0.05
	After 8 injections	1:1280	0.0125	1:1280	0.05	1:640	0.006	1:1280	0.0125
	After 9 injections	1:1280	0.0125	1:1280	0.025	1:1280	0.006	1:1280	0.0125
	After 11 injections	1:1280	0.0125	1:640	0.025	1:1280	0.003	1:1280	0.003
No. 2	After 4 doses	1:320	trace	1:320	0.05	1:160	trace	1:640	0.05
No. 3	After 6 doses	1:640	0.05	1:640	0.05	1:320	0.025	1:640	0.025
No. 4	After 6 doses	1:320	0.003	1:320	0.003	1:320	0.003	1:640	0.00125
No. 5	After 7 doses	1:1280	0.003	1:640	0.003	1:640	0.003	1:640	0.006

CONCLUSIONS

1. The immunization of rabbits with human corpuscles belonging to groups I, II, III and IV does not result in the production of specific agglutinins and hemolysins for the corpuscles of the group employed in immunization. These sera, however, frequently show slightly more agglutinin and hemolysin for the corpuscles of the group used in immunization than for the corpuscles of the remaining groups.
2. Absorption of these immune sera to remove the group agglutinins and hemolysins have generally resulted in the removal of all agglutinins and hemolysins.
3. For the grouping of human erythrocytes it does not appear possible to prepare specific immune sera; human sera containing isoagglutinins and hemolysins must be used and these are best preserved in a fluid state at or near the freezing point.

We beg to acknowledge the assistance rendered by Mr. Joseph Sands in the conduct of a part of this investigation.

REFERENCES

(1) Kolmer, J. A., Trist, M. E., and Flick, A. M.: A study of the natural thermolabile and thermostabile hemolysins and hemagglutinins in human serum in relation to the Wassermann reaction. Amer. Jour. of Syphilis, 1920, 4, 111-135.

(2) Karsner, H. T., and Koeckert, H. L.: The influence of desiccation on human normal isohemagglutinins. Jour. Amer. Med. Assoc., 1919, 73, 1207–1210.

(3) Sanford, A. H. A modification of the Moss method of determining isohemagglutination groups. Jour. Amer. Med. Assoc., 1918, 70, 1221.

(4) Kolmer, J. A., and Brown, C. P.: The red corpuscle suspension for the Wassermann reaction. The preservation of red blood cells. Amer. Jour. of Syphilis, 1919, 3, 169-193.

(5) Kolmer, J. A.: The influence of desiccation on human normal isohemagglutinins. Jour. Amer. Med. Assoc., 1919, 73, 1459–1460.

A COMPARATIVE STUDY OF METHODS FOR THE PREPARATION OF TYPHOID AGGLUTINOGENS

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Various methods have been proposed for the preparation of antigens for macroscopic agglutination tests, most attention having been given those prepared of bacilli of the typhoid-paratyphoid group and *B. mallei;* in the various methods proposed, broth cultures and suspensions of the microörganisms washed from solid media in saline solution, have been usually employed. In some methods, the antigen is heated for sterilization and in others it is used unheated; various chemicals have been employed for sterilization, when heat has not been applied, and also for preservation, including formalin (as in the Dryer method), phenol and tricresol, in varying amounts.

PURPOSE OF INVESTIGATION

A systematic study of the influence of preparation upon the qualities of an agglutinogen, with particular reference to the influence of heat and the kind and more particularly the amount of chemical germicide and preservative employed, does not appear to have been made, although it is well known that the technic employed modifies the results to be observed in agglutination tests, aside from variations due to the microörganism itself. At the suggestion of Professor Kolmer, agglutinogens of a single strain of *B. typhosus* have been prepared after various methods and tested against the sera of rabbits immunized with the same culture, to determine the influence of technic of preparation upon the qualities of agglutinogens of this bacillus, that is, regarding spontaneous agglutination and susceptibility to specific agglutination by the immune sera.

TECHNIC EMPLOYED

Culture. The culture of *B. typhosus* employed throughout this investigation has been used in microscopical agglutination tests for many years and found quite susceptible to specific agglutinins and usually free of spontaneous agglutination. All agglutinogens were prepared of this one strain, and all rabbits were immunized with the same, being injected intravenously at intervals of five to seven days with increasing doses of living bacilli.

Agglutinogens. Twenty-one agglutinogens were used in this study; the first six were prepared from a ninety-six hour culture in plain neutral broth as follows:

No. 1. Heated at 60°C. for two hours and preserved with 0.5 per cent phenol.

No. 2. Heated at 60°C. for two hours; no preservative.

No. 3. Heated at 60°C. for two hours; preserved with 0.5 per cent phenol and 10 per cent glycerin.

No. 4. Not heated; preserved with 0.5 per cent phenol.

No. 5. Not heated; preserved with 0.5 per cent tricresol.

No. 6. Not heated; preserved with 1:2000 mercurophen.[1]

Agglutinogens 7 to 16 were prepared from a suspension of bacilli secured by growing the strain on plain neutral agar in a large series of Blake bottles for forty-eight hours, removing with physiological saline solution, shaking the emulsion with beads until a uniform suspension was secured and diluting with sufficient saline until each cubic centimeter contained about 2,000,000,000 bacilli.

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[1] Mercurophen is the name applied to sodium oxy-mercury-orthro-nitro phenolate, a new and superior mercurial germicide prepared by Schamberg, Kolmer and Raiziss, Jour. Infect. Dis., 1919.

No. 7. Unheated; preserved with 0.1 per cent formalin.
No. 8. Unheated; preserved with 0.5 per cent formalin.
No. 9. Unheated; preserved with 1.0 per cent formalin.
No. 10. Unheated; preserved with 2.0 per cent formalin.
No. 11. Unheated; preserved with 5.0 per cent formalin.
No. 12. Unheated; preserved with 1.0 per cent phenol.
No. 13. Unheated; preserved with 5.0 per cent phenol.
No. 14. Unheated; preserved with 1.0 per cent tricresol.
No. 15. Unheated; preserved with 1:1000 mercurophen.
No. 16. Unheated; preserved with 1:5000 mercurophen.

Agglutinogens 17 to 21 were freshly prepared as required by removing forty-eight hour growths from slants of plain neutral agar with the following fluids; these suspensions were treated as agglutinogens 7 to 16, except that preservatives were not added:

No. 17. Unheated; suspension in 0.85 per cent sodium chloride in distilled water.
No. 18. Unheated; suspension in 1 per cent sodium chloride in distilled water.
No. 19. Unheated; suspension in 2 per cent sodium chloride in distilled water.
No. 20. Unheated; suspension in 5 per cent sodium chloride in distilled water.
No. 21. Unheated; suspension in distilled water.

Antigens 1 to 16 were kept in a refrigerator near the freezing point and first tested with immune sera about one week after preparation and again about one month later; as previously stated antigens 17 to 21 were fresly prepared as required.

Density of agglutinogens. The strain of *B. typhosus* employed cultivated for ninety-six hours in extract broth neutral to phenolphthalin, produced a culture of just sufficient density for the technic employed which consisted in setting up the tests in tubes having an internal diameter of 1 cm. and using each agglutinogen in amount of 1 cc. with 1 cc. of diluted serum. Suspensions made of cultures grown on solid media and diluted so that each cubic centimeter finally contained about 2,000,000,-

000 bacilli, proved just about right in the tests employed. Denser cultures very much obscured the results and rendered the readings difficult and uncertain; thinner cultures were likewise unsatisfactory and one *general result of the study was to show the technical importance of proper density of cultures and the necessity of having this factor satisfactorily adjusted to the size of the tubes and total volume of fluid employed.*

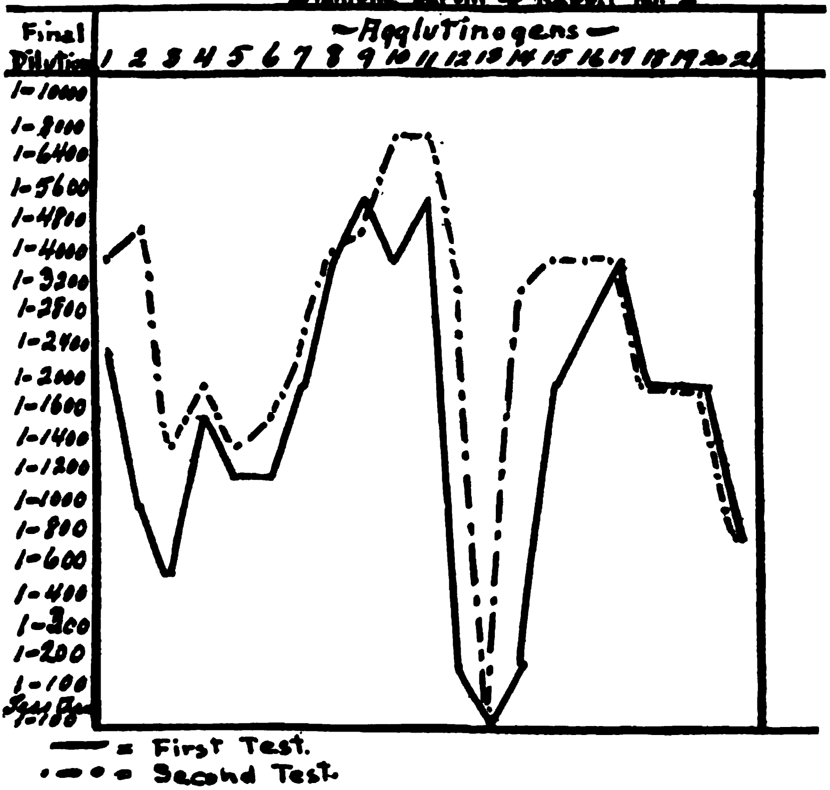
Technic. A uniform macroscopic technic was employed throughout; sera were diluted with sterile physiological saline solution (0.85 per cent chemically pure sodium chloride in distilled water) and used in appropriate test tubes in amounts of 1 cc.; each antigen was used in dose of 1 cc., thereby doubling each dilution. Controls on each antigen were included in each experiment. All tubes were incubated at 55°C. for about twenty-four hours and readings were made with the naked eye; generally readings were made by two persons in order to reduce the error due to the personal equation in the interpretation of results.

The sera of five rabbits were employed with each agglutinogen. In the first tests the sera were used after each rabbit had received four intravenous injections of the culture; the second series of tests were conducted with sera after five injections and the third series after eight injections. In each experiment the sera were so diluted as to obtain the limits of agglutination with each antigen.

RESULTS

The results were recorded according to the maximum dilution of each serum in which agglutination was detectable with the naked eye, namely, the production of flocculi with or without some sediment, and for brevity they are recorded graphically in charts 1 to 5. These charts present the results observed with the serum of each rabbit in two out of three sets of reactions with all agglutinogens, and they show the marked influence the method of preparation excited upon the results of agglutination tests.

Chart I Curves of Agglutination Observed with the Immune Serum of Rabbit no. I



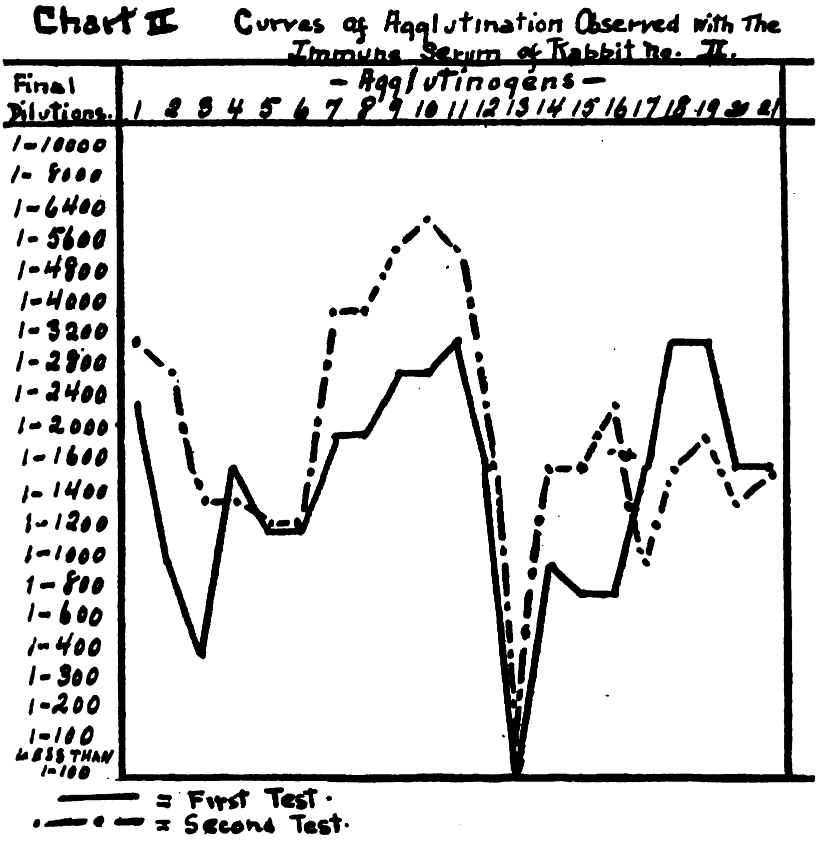


Chart II Curves of Agglutination Observed with The Immune Serum of Rabbit No. II.
Final Dilutions.
– Agglutinogens –
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
1-10000
1-8000
1-6400
1-5600
1-4800
1-4000
1-3200
1-2800
1-2400
1-2000
1-1600
1-1400
1-1200
1-1000
1-800
1-600
1-400
1-300
1-200
1-100
Less than 1-100
= First Test.
= Second Test.

Chart III Curves of Agglutination Observed with the Immune Serum of Rabbit No. III.

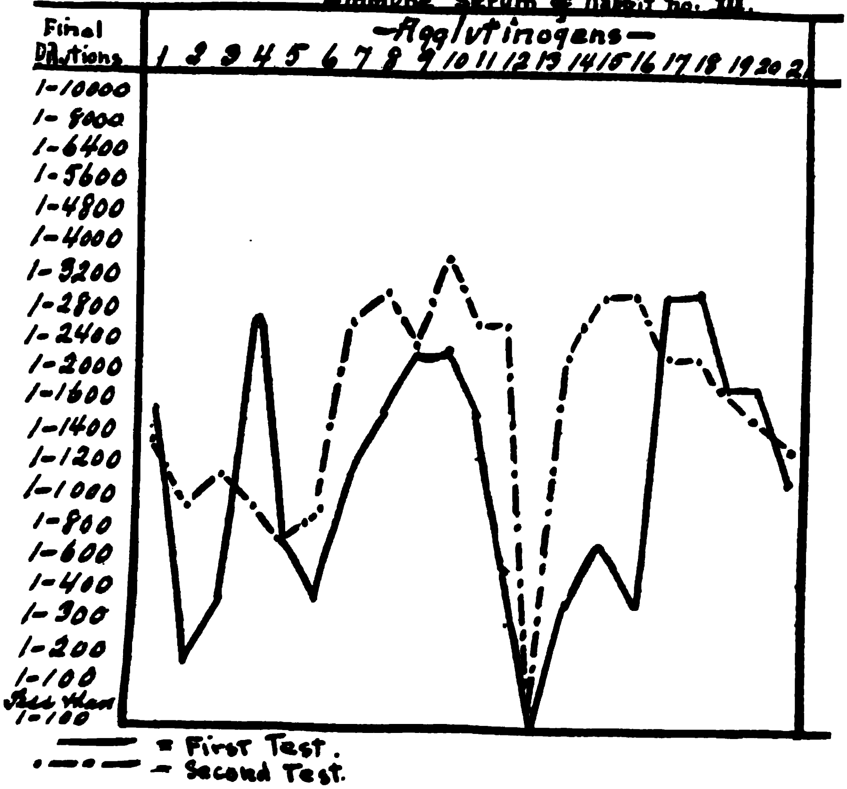


Chart IV. Curves of Agglutination Observed with the Immune Serum of Rabbit no. IV.

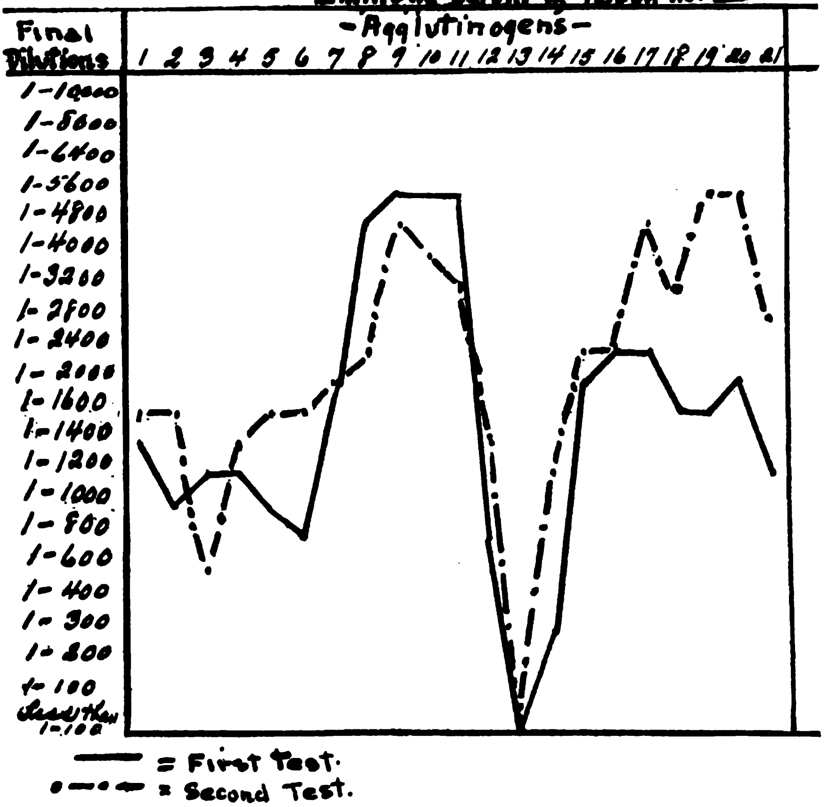
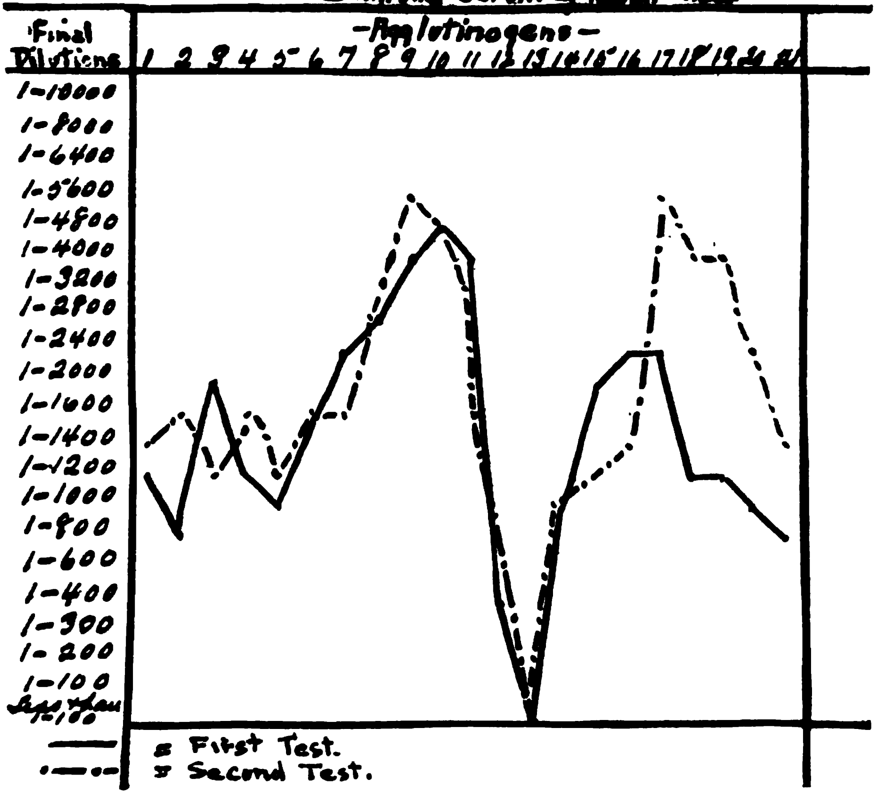


Chart V. Curves of Agglutination Observed with the Immune Serum of Rabbit No. V.



SUMMARY

1. Agglutinogens of broth cultures vs. saline suspensions. Of primary importance are the results in relation to agglutinogens prepared by cultivation of the microörganism in broth as compared with those prepared by cultivating on plain solid media and suspending the bacteria in saline solution: a general review of the results observed in this study with a single strain of typhoid bacilli, indicate that suspensions in saline solution so prepared as to break up clumps and diluted to proper density, are superior to broth cultures.

2. Saline solutions vs. distilled water. As is well known, sodium chloride exerts an important rôle in the physicochemical phenomenon of agglutination; of agglutinogens prepared by suspending typhoid bacilli in strengths of sodium chloride in sterile distilled water varying from 0.85 to 5 per cent (nos. 17, 18, 19 and 20), best results were observed with the 0.85 and 1 per cent solutions (nos. 17 and 18); agglutinogens prepared with 2 per cent solutions of sodium chloride were less susceptible to agglutination and 5 per cent solutions were decidedly less susceptible. Agglutinogens prepared with distilled water alone (no. 21) were least susceptible to agglutination and yielded some of the lowest titers.

3. Influence of heat. Of primary importance is the effect of heating an agglutinogen upon its susceptibility to agglutination; in the majority of laboratories cultures or suspensions are usually heated at 56° to 60°C. for one-half to two hours. In order to be able to observe the most marked influence of heat, if any, antigens were heated in a water bath at 60°C. for two hours; a general survey of the results of this study indicates that heated antigens are somewhat more susceptible to agglutination than unheated antigens.

4. Influence of chemical germicides and preservatives. In this investigation phenol, tricresol, formalin, mercurophen and glycerin were employed for chemical sterilization and preservation (antiseptic activity); with the exception of formalin in 0.1 to 5 per cent; agglutinogens prepared without preservatives were somewhat superior to those containing phenol, tricresol, mercurophen and glycerin.

5. Kind of chemical germicide and preservative. Of the chemicals employed in the preparation of agglutinogens of *B. typhosus, best results were observed with formalin;* in fact the addition of 1 to 2 per cent formalin[3] to suspensions in isotonic saline solution yielded the best agglutinogens of the series included in this study (nos. 9 and 10). Agglutinogens were prepared with 0.1, 0.5, 1, 2 and 5 per cent neutral formalin; comparative tests have usually shown that those containing 1 and 2 per cent were least likely to show spontaneous agglutination, were most susceptible to specific serum agglutination and were never contaminated.

Agglutinogens prepared with phenol in 0.5 per cent and tricresol in 0.5 per cent (nos. 4 and 5) yielded similar results, but they were somewhat inferior to preservative free agglutinogens and decidedly inferior to those containing 0.1 to 2 per cent formalin. Agglutinogens prepared with 1 per cent phenol and tricresol (nos. 12 and 14) were decidedly inferior to those containing 0.5 per cent of these substances and an antigen containing 5 per cent phenol (no. 13) was almost insusceptible to agglutination and proved most unsatisfactory of all.

Mercurophen was employed because of its high germicidal activity and freedom of precipitating and coagulating influence upon proteins including bacterial proteins; agglutinogens prepared with 1:1000 mercurophen in physiological saline solution (no. 15) and 1:2000 (no. 6) yielded results similar to those containing 0.5 per cent phenol and tricresol. An agglutinogen containing 1:5000 mercurophen (no. 16) was generally satisfactory and from the standpoints of freedom from spontaneous agglutination, susceptibility to specific agglutination and freedom from contamination ranked next to plain and formalized agglutinogens.

The addition of 10 cc. of the best grade neutral glycerin to each 100 cc. of heated agglutinogen containing 0.5 per cent phenol (no. 3) reduced susceptibility to specific agglutination and proved inferior to the same agglutinogen prepared without the addition of glycerin (no. 1).

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[3] The formalin used contained 39.2 per cent formaldehyde gas; the 1 per cent solution, therefore, contained 0.39 per cent of formaldehyde.

6. *Spontaneous agglutination.* Of agglutinogens 1 to 16 preserved over a period of four to eight weeks in a refrigerator, no. 15 containing 1: 1000 mercurophen, showed most tendency to spontaneous agglutination and nos. 7, 8, 9 and 10 containing 0.1 to 2 per cent formalin, least spontaneous agglutination; of the freshly prepared antigens, nos. 19 and 20, containing 2 and 5 per cent sodium chloride frequently showed spontaneous agglutination. Macroscopical tests for spontaneous agglutination were conducted with each antigen whenever employed by diluting 1 cc. with 1 cc. of 0.85 per cent saline solution and incubating at 55°C. for twenty-four hours; at the same time microscopical tests were made and occasionally antigens showed small clump of bacilli microscopically, which appeared perfectly homogenous and satisfactory to the closest scrunity with the naked eye.

CONCLUSIONS

1. A comparative study of agglutinogens prepared from a single strain of typhoid bacilli which had been used for agglutination tests for several years, was made by comparing their susceptibility to specific agglutination by rabbit immune sera, tendency to spontaneous agglutination, keeping qualities and susceptibility to contamination.

2. The density of the agglutinogen was found to have an important influence, regardless of the method of preparation; thick suspensions obscured results and reactions while very thin suspensions were difficult to read with the naked eye. The density of a particular agglutinogen should be adjusted according to the diameter of the test tubes employed and total volume of fluid.

3. Suspensions in saline solution of microörganisms washed from solid media, were generally superior to broth cultures.

4. The best saline solutions for the preparation of agglutinogens were found to be those containing 0.85 to 1 per cent chemically pure sodium chlorid in distilled water.

5. Distilled water alone was found unsatisfactory for the preparation of typhoid agglutinogen.

6. Heating an agglutinogen at 60°C. for two hours generally increased its susceptibility to specific agglutinins.

7. Agglutinogens prepared without preservatives with the exception of those preserved with formalin were generally superior to those containing phenol, tricresol, mercurophen and glycerin.

8. The best agglutinogens were found to be those containing 1 to 2 per cent formalin.

9. The addition of more than 0.5 per cent phenol and tricresol to an agglutinogen reduced its susceptibility to specific serum agglutinins; the addition of glycerin also reduced the susceptibility to specific agglutination.

10. An agglutinogen of typhoid bacilli is best prepared by cultivating on solid media for forty-eight hours, removing the growths with 0.85 to 1 per cent chemically pure sodium chlorid in distilled water, shaking with beads until a perfectly homogenous emulsion is secured, diluting with saline solution to proper density (about 2,000,000,000 per cubic centimeter), and adding neutral formalin to 1 per cent.

The writer wishes to express his sincere gratitude to Prof. John A. Kolmer for his very kind assistance throughout the entire course of this study; he is also indebted to Dr. M. Matsumato and Dr. Yosiho Saeki for their aid in the preparation of the agglutinogens.

A STUDY OF DIFFERENT METHODS FOR THE PREPARATION OF B. TYPHOSUS ANTIGEN

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One of the reasons generally assigned for the unsatisfactory status of complement-fixation tests in the diagnosis of bacterial infections, is the difficulty of preparing efficient and stable antigens; among the diseases of bacterial origin complement-fixation is probably most widely employed in the diagnosis of glanders, tuberculosis and gonococcus infections, but in the last mentioned the test is well known as lacking in the sufficient delicacy and it probably can be rendered more sensitive by further improvement of the antigen, and the same may be true of the tuberculosis complement-fixation test.

Bacterial antigens for complement-fixation tests may be divided into four main groups, namely (a) those composed of whole bacteria and their soluble products in the fluid medium in which they have been cultivated; (b) those in which the bacterial cells alone are utilized suspended in sterile salt solution; (c) those in which the bacterial cells are disrupted but not filtered and (d) those in which the cells are disrupted and the insoluble portions removed by filtration, the filtrate being employed as antigen. The antigen commonly employed for the gonococcus complement-fixation test may be classified under the last mentioned in which the soluble intracellular substances are utilized as antigen; in a study of these gonococcus antigens Kolmer and Brown (1) found that whole suspensions of gonococci in saline solution classified under (b) above, proved superior in antigenic sensitiveness to filtrates, and similar results were observed in complement fixation tests in typhoid fever (2), diphtheria (3) and canine distemper (4).

PURPOSES OF INVESTIGATION

In view of the practical importance of increasing the sensitiveness and delicacy of complement fixation in bacterial infections, Professor Kolmer suggested a further systematic and comparative study of prevailing methods for the preparation of bacteria antigens taking a single pure culture of *B. typhosus* as the test microörganism and preparing antigens after the four main varieties described above; a secondary object was the study of complement fixation in typhoid fever and after active immunization of persons with typhoid vaccine, as an additional means for comparing the antigenic sensitiveness of the various antigens.

Each antigen has been studied for its anticomplementary, hemolytic and antigenic values; the antigenic titrations were conducted with the sera of rabbits immunized with the same strain as used in the preparation of the antigens and also with the sera of persons containing typhoid antibodies. The results are summarized in this communication.

PREPARATION OF ANTIGENS

Antigen 1. This antigen was composed of living bacilli suspended in physiological saline solution and freshly prepared as required by removing twenty-four hour cultures on plain neutral agar with saline and shaking with sterile glass beads until a homogenous suspension was secured.

Antigen 2. This antigen was a forty-eight hour growth in plain beef extract broth neutral to phenolphthalein, shaken mechanically for an hour to secure a homogenous suspension followed by heating in a water-bath at 60°C. for one one hour and preservation in a refrigerator with 0.5 per cent phenol.

Antigen 3. The same as antigen II except that a fourteen-day broth culture was employed.

The remaining six antigens were prepared from mass cultures of the strain of *B. typhosus* removed from a large series of agar cultures in Blake bottles, due care being utilized against removing bits of culture medium. In order to make sure that agar and other bacteria were not included, the bacterial mass was briefly centrifuged and cultured before use.

Antigen 4. Five hundred cubic centimeters of a heavy suspension of bacilli in sterile distilled water was heated in a water-bath at 56°C. for one hour, then at 80°C. for an hour. The heated suspension was shaken mechanically for twenty-four hours after which treatment it was centrifuged and the supernatant fluid was passed through sterile neutral porcelain filters. The filtrate was then heated at 56°C. for one hour on three successive days and preserved in a refrigerator with 0.5 per cent. phenol.

Antigen 5. A saline suspension of bacilli was thoroughly centrifuged and the sediment was dried over calcium chlorid; each 0.05 gram of dried bacterial mass was ground into a very fine powder and gradually suspended in 25 cc. of physiological saline solution. This emulsion was shaken mechanically for twenty-four hours and passed through a porcelain filter and the filtrate was preserved in a refrigerator for antigen.

Antigen 6. Five hundred cubic centimeters of a heavy saline suspension of bacilli was precipitated with an equal quantity of absolute ethyl alcohol and thoroughly centrifuged; the sediment was dried over calcium chlorid, ground into a fine powder, weighed and suspended in sufficient saline solution to make a 2 per cent. emulsion. The resulting product was quite thick and required further dilution with saline prior to use.

Antigen 7. This antigen was prepared after the method described by Hitchens and Hansen (5) for the preparation of meningococcus antigen; briefly the technic consisted of precipitation of 500 cc. of a heavy suspension of bacilli in distilled water with an equal amount of 95 per cent. ethyl alcohol and thoroughly centrifuged at once for the sediment; the sediment was resuspended in alcohol and again centrifuged, this process being repeated several times with alcohol and finally several times with ethyl ether. The final sediment was freed of ether, dried over calcium chlorid and ground to a very fine powder and 0.02 gram was suspended in 20 cc. of sterile saline solution for antigen as required.

Antigen 8. This antigen was prepared after the method described by Small (6); briefly the technic consisted in thoroughly centrifuging a heavy saline suspension of bacilli and drying the bacterial sediment at 56°C.; 0.5 gram of this powder was moistened with chloroform and thoroughly ground with the addition of a small amount of ether from time to time until a very fine dry powder was obtained. This powder was now suspended in a mixture of equal parts of chloroform and ether and shaken mechanically for six hours followed by several washings of the sediment with ether and drying of the ether moist residue at 56°C.; when used about 0.5 gram of the powder was suspended in 25 cc. of saline solution and further diluted with saline solution.

Antigen 9. This antigen was prepared after the method described by Miss Wilson (7) for the preparation of antigen of tubercle bacilli for the complement fixation test; 2000 cc. of a five day culture of the strain of *B. typhosus* in plain neutral broth was heated in an Arnold sterilizer for one hour and thoroughly centrifuged for the bacilli; the sediment was then treated a number of times with ten volumes of absolute ethyl alcohol and finally with ether, the sediment being secured each time by centrifuging. After the final treatment with ether the bacterial sediment was dried at room temperature, ground into a fine powder, weighed as required and prepared in a 0.5 per cent suspension in sterile saline solution.

TECHNIC

Titrations for anticomplementary power. Each antigen was titrated at intervals and just prior to complement-fixation tests with immune sera, for its anticomplementary or antilytic value, the smallest amount producing the slightest inhibition of hemolysis being taken as the anticomplementay unit. All antigens were titrated at the same time and with the same hemolytic system in order to render the results strictly comparative.

In conducting these titrations the antigens were used undiluted or diluted with saline solution as required and placed in a series of 12 test tubes in amounts ranging from 0.02 to 2 cc.; complements were furnished by the mixed sera of guinea-pigs in a dose of 0.5 cc. of 1:20 dilution. After salt solution had been added to each tube carrying antigen and complement to bring the total volume to 2.5 cc., incubation was conducted in a water-bath at 38°C. for one hour and this was followed by the addition of two units of antisheep hemolysin and 0.5 cc. of a 2.5 per cent suspension of sheep corpuscles; the tubes were then reincubated for an hour and placed in a refrigerator over night, the results being read the next morning. The usual hemolytic, complement and corpuscle controls were included.

Hemolytic titrations. By including relatively large doses of each antigen in the anticomplementary titrations the direct hemolytic dose of each in the presence of complement was generally obtained and served as a means for comparing the hemolytic activity of the various preparations.

Antigenic titrations. These were conducted with sera of immunized rabbits and of persons having had typhoid fever or active immunization with typhoid vaccine. Rabbits were immunized with the same culture of *B. typhosus* employed in the preparation of the antigens, increasing doses of heated killed and finally living bacilli being injected intravenously and the sera obtained from these animals were employed in the complement fixation tests when the agglutinins had reached a titer somewhat comparable to their concentration in the sera of persons during typhoid fever or after a course of injections of typhoid vaccine.

In order to avoid the non-specific fixation of complement by rabbit sera described by Kolmer and his associates (8), each serum was used unheated or after heating at 62°C. in a water bath for thirty minutes and in small amounts ranging from 0.0001 to 0.02 cc. Human sera were used unheated and after heating at 56°C. for thirty minutes in amounts ranging from 0.01 to 0.1 cc.

In conducting the antigen titrations with immune sera to bring out the differences in antigenic sensitiveness of the various preparations the following method was employed; each antigen was used in a constant dose equal to one-third its anticomplementary unit in a series of twelve test tubes and unheated rabbit sera added in amounts ranging from 0.0001 cc. to 0.02 cc.; a serum control on each serum was included in which 0.02 cc. serum alone was used. A control on each antigen was included as likewise hemolytic, complement and corpuscle controls. Complement was furnished by the mixed sera of guinea-pigs in a constant dose of 0.5 cc. of 1:20 dilutions and saline solution was added to bring the total volume in each tube to 1 cc. Primary incubation was conducted at 38°C. in a water-bath for one hour and this was followed by the addition of two units of hemolysin and 0.5 cc. of 2.5 per cent sheep cells; after reincubation for an hour the results were read after the tubes had been placed in a refrigerator overnight.

Agglutination tests. The agglutinin content of each human and rabbit serum for the strain of *B. typhosus* employed in this work was determined in a macroscopic test with the employment of living suspensions and an incubation at 38°C. for one hour; the results were read by the naked eye after the tubes had been placed in a refrigerator over night.

RESULTS

a. Anticomplementary and keeping qualities of the different antigens. The results of anticomplementary titrations with freshly prepared antigens are shown in table 1; these titrations were repeated at subsequent periods and the results are summarized in table 2, the smallest amount of each antigen producing slight inhibition of hemolysis being registered as the anticomplementary unit.

None of the antigens were markedly anticomplementary but a strict comparison could not be made in-as-much as this would have required the preparation of antigens according to numerical numbers of bacilli entering into a uniform given volume of each preparation.

All of the antigens were preserved in a refrigerator near the freezing point and the series of titrations of anticomplementary power over a period of six weeks following their preparation

TABLE 1

The first anticomplementary titration of antigens

DOSE OF ANTIGEN	ANTIGENS								
	No. 1 Undiluted	No. 2 Undiluted	No. 3 Undiluted	No. 4 Undiluted	No. 5 1:10	No. 6 1:10	No. 7 1:10	No. 8 1:50	No. 9 1:10
cc.									
0.02	C.H.*	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.04	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.06	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.08	C.H.	C.H.	M.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.1	C.H.	C.H.	M.H.	C.H.	C.H.	C.H.	C.H.	C.H.	M.H.
0.2	M.H.	C.H.	N.H.	M.H.	C.H.	C.H.	C.H.	C.H.	M.H.
0.4	S.H.	C.H.	N.H.	S.H.	C.H.	M.H.	C.H.	C.H.	S.H.
0.6	N.H.	S.H.	N.H.	N.H.	C.H.	M.H.	C.H.	C.H.	N.H.
0.8	N.H.	N.H.	N.H.	N.H.	C.H.	C.H.	C.H.	C.H.	N.H.
1.0	N.H.	N.H.	M.H.	N.H.	C.H.	C.H.	C.H.	C.H.	N.H.
1.5	N.H.	N.H.	C.H.	C.H.	M.H.	C.H.	M.H.	M.H.	N.H.
2.0	N.H.	N.H.	C.H.	C.H.	M.H.	C.H.	S.H.	S.H.	N.H.

* C.H., Complete hemolysis; M.H., marked hemolysis, the anticomplementary unit; S.H., slight hemolysis; N.H., no hemolysis.

TABLE 2

Summary showing anticomplementary units of each antigen as determined at varying intervals

	*FIRST TITRATION	SECOND TITRATION	THIRD TITRATION	FOURTH TITRATION
Antigen 1....................	0.2†	0.6	0.4	0.3
Antigen 2....................	0.6	0.6	0.6	0.5
Antigen 3....................	0.15	0.2	0.2	0.3
Antigen 4....................	0.2	0.2	0.4	0.2
Antigen 5....................	0.15	0.3		
Antigen 6....................	0.04	0.04	0.06	0.05
Antigen 7....................	0.15	0.4	0.1	0.06
Antigen 8....................	0.03	0.03	0.06	0.07
Antigen 9....................	0.01	0.04	0.02	0.01

* First titration conducted with freshly prepared antigens; second titration conducted ten days later; third titration conducted one week after the second and fourth titration about six weeks after the third.

† Amount in cubic centimeters of undiluted antigen producing slight inhibition of hemolysis.

TABLE 3

Comparative antigenic sensitiveness of antigens with active serum of rabbit 1 (agglutination titer 1: 100)

DOSE OF SERUM	ANTIGENS USED IN ONE-THIRD ANTICOMPLEMENTARY UNITS								
	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6	No. 7	No. 8	No. 9
cc.									
0.0001	−	−	−	−	−	−	−	−	−
0.0002	−	−	−	−	−	−	−	−	−
0.0004	−	−	++	−	−	−	−	−	−
0.0006	+	−	++	−	−	−	−	−	−
0.0008	+	−	++	−	−	−	−	+	++
0.001	++	+	+++	−	−	±	+	+++	+++
0.002	++++	+++	++++	−	−	++	++	++++	+++
0.004	++++	++++	++++	−	++	++++	++++	++++	++++
0.006	++++	++++	++++	+	++++	++++	++++	++++	++++
0.008	++++	++++	++++	+++	++++	++++	++++	++++	++++
0.01	++++	++++	++++	+++	++++	++++	++++	++++	++++
0.02	++++	++++	++++	++++	++++	++++	++++	++++	++++

− Negative; ± doubtfully positive; + very weakly positive; ++ weakly positive; +++ , moderately positive; ++++ strongly positive.

TABLE 4

Comparative antigenic sensitiveness of antigens with active serum of rabbit 2 (agglutination titer 1: 80)

DOSE OF SERUM	ANTIGEN USED IN ONE-THIRD ANTICOMPLEMENTARY UNITS								
	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6	No. 7	No. 8	No. 9
cc.									
0.0001	−	−	−	−	−	−	−	−	−
0.0002	−	−	−	−	−	−	−	−	−
0.0004	−	−	−	−	−	−	−	−	−
0.0006	−	−	±	−	−	−	−	−	−
0.0008	±	−	+	−	−	−	−	−	−
0.001	+	±	++	−	−	−	−	−	−
0.002	+++	++	+++	−	−	±	+	+	+++
0.004	++++	+++	++++	−	+	+	++	+++	++++
0.006	++++	++++	++++	±	+++	+++	+++	++++	++++
0.008	++++	++++	++++	+	++++	++++	++++	++++	++++
0.01	++++	++++	++++	+++	++++	++++	++++	++++	++++
0.02	++++	++++	++++	++++	++++	++++	++++	++++	++++

− negative; ± doubtfully positive; + very weakly positive; ++ weakly positive; +++ moderately positive; ++++ strongly positive.

TABLE 5

Comparative antigenic sensitiveness of antigens with active serum of rabbit 3 (agglutination titer 1: 60)

DOSE OF SERUM	ANTIGEN USED IN ONE-THIRD ANTICOMPLEMENTARY UNITS							
	No. 1	No. 2	No. 3	No. 4	No. 6	No. 7	No. 8	No. 9
cc.								
0.0001	–	–	–	–	–	–	–	–
0.0002	–	–	–	–	–	–	–	–
0.0004	–	–	–	–	–	–	–	–
0.0006	–	–	–	–	–	–	–	–
0.0008	–	–	–	–	–	–	–	–
0.001	–	–	–	–	–	–	–	–
0.002	+	–	++	–	±	–	–	–
0.004	++	±	++++	–	+	–	+	–
0.006	+++	+	++++	–	+++	–	++	+
0.008	+++	++	++++	–	+++	±	+++	++
0.01	++++	+++	++++	–	++++	+	+++	+++
0.02	++++	++++	++++	+	++++	++++	++++	+++

– negative; ± doubtfully positive; + very weakly positive; ++ weakly positive; +++ moderately positive; ++++ strongly positive.

TABLE 6

Comparative antigenic sensitiveness of antigens with heated serum of rabbit 3 (agglutination titer 1: 60)

DOSE OF SERUM	ANTIGENS USED IN ONE-THIRD ANTICOMPLEMENTARY UNITS							
	No. 1	No. 2	No. 3	No. 4	No. 6	No. 7	No. 8	No. 9
cc.								
0.0001	–	–	–	–	–	–	–	–
0.0002	–	–	–	–	–	–	–	–
0.0004	–	–	–	–	–	–	–	–
0.0006	–	–	–	–	–	–	–	–
0.0008	–	–	–	–	–	–	–	–
0.001	–	–	±	–	–	–	–	–
0.002	±	–	+	–	–	–	–	–
0.004	++	–	+++	–	–	–	±	–
0.006	++++	++++	++++	0	+++	0	0	0
0.008	++++	++++	++++	–	+++	–	++	±
0.01	++++	++++	++++	–	++++	±	+++	+
0.02	++++	++++	++++	++	++++	+++	++++	++++

– negative; ± doubtfully positive; + very weakly positive; ++ weakly positive; +++, moderately positive; ++++ strongly positive; 0 lost by accident.

TABLE 7

Comparative antigenic sensitiveness of antigens with active serum of rabbit 4 (agglutination titer 1: 240)

DOSE OF SERUM	ANTIGENS USED IN ONE-THIRD ANTICOMPLEMENTARY UNITS							
	No. 1	No. 2	No. 3	No. 4	No. 6	No. 7	No. 8	No. 9
cc.								
0.0001	−	−	−	−	−	−	−	−
0.0002	−	−	−	−	−	−	−	−
0.0004	−	−	−	−	−	−	−	−
0.0006	+	−	−	−	−	−	−	−
0.0008	++	−	+	−	−	−	−	−
0.001	+++	−	+++	−	−	−	−	−
0.002	+++	+++	++++	−	+	−	+	−
0.004	++++	++++	++++	−	++++	+	++++	+
0.006	++++	++++	++++	±	++++	++++	++++	+++
0.008	++++	++++	++++	+	++++	++++	++++	++++
0.01	++++	++++	++++	++	++++	++++	++++	++++
0.02	++++	++++	++++	+++	++++	++++	++++	++++

− negative; ± doubtfully positive; + very weakly positive; ++ weakly positive; +++ moderately positive; ++++ strongly positive.

TABLE 8

Comparative antigenic sensitiveness of antigens with heated serum of rabbit 4 (agglutination titer 1: 240)

DOSE OF SERUM	ANTIGENS USED IN ONE-THIRD ANTICOMPLEMENTARY UNITS							
	No. 1	No. 2	No. 3	No. 4	No. 6	No. 7	No. 8	No. 9
cc.								
0.0001	−	−	−	−	−	−	−	−
0.0002	−	−	−	−	−	−	−	−
0.0004	−	−	−	−	−	−	−	−
0.0006	±	−	−	−	−	−	−	−
0.0008	+	−	±	−	−	−	−	−
0.001	++	−	++	−	−	−	−	−
0.002	+++	+	++++	−	+	−	+	−
0.004	++++	++++	++++	−	++++	±	+++	−
0.006	++++	++++	++++	−	++++	++++	++++	+++
0.008	++++	++++	++++	−	++++	++++	++++	++++
0.01	++++	++++	++++	−	++++	++++	++++	++++
0.02	++++	++++	++++	+	++++	++++	++++	++++

− negative; ± doubtfully positive; + very weakly positive; ++ weakly positive; +++ moderately positive; ++++ strongly positive.

TABLE 9

Summary showing smallest amount of each unheated and heated rabbit immune serum giving ++++ with one-third the anticomplementary unit of each antigen

ANTIGENS	UNHEATED RABBIT SERA				HEATED RABBIT SERA	
	Rabbit 1	Rabbit 2	Rabbit 3	Rabbit 4	Rabbit 3	Rabbit 4
No. 1..........	0.002	0.004	0.01	0.004	0.006	0.004
No. 2..........	0.004	0.006	0.02	0.004	0.006	0.004
No. 3..........	0.002	0.005	0.004	0.002	0.006	0.002
No. 4..........	0.02	0.02	0	0	0	0
No. 5..........	0.006	0.008	0	0	0	0
No. 6..........	0.004	0.008	0.01	0.004	0.01	0.004
No. 7..........	0.004	0.008	0.02	0.006	0	0.006
No. 8..........	0.002	0.006	0.02	0.004	0.02	0.006
No. 9..........	0.004	0.004	0	0.008	0.02	0.008

Agglutination titer of rabbit 1 was 1:100, rabbit 2, 1:80; rabbit 3, 1:60; rabbit 4, 1:240.

TABLE 10

Results with the unheated serum of a person in the fourth week of typhoid fever; agglutination titer 1: 40

DOSE OF SERUM	RESULTS WITH DIFFERENT ANTIGENS								
	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6	No. 7	No. 8	No. 9
cc.									
0.01	++++	+	++++	−	−	−	+	++++	++++
0.02	++++	+	++++	−	−	−	+	++++	++++
0.04	++++	++	++++	−	−	−	++	++++	++++
0.06	++++	+++	++++	−	−	−	++	++++	++++
0.08	+++	+++	++++	−	−	−	+++	+++	+++
0.1	+++	+++	++++	−	−	−	+	+++	+++

TABLE 11

Results with the unheated serum of a person in the sixth week of typhoid fever; agglutination titer 1: 320

DOSE OF SERUM	RESULTS WITH DIFFERENT ANTIGENS								
	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6	No. 7	No. 8	No. 9
cc.									
0.01	++++	++++	++++	++++	++++	++++	++++	++++	++++
0.02	++++	++++	++++	++++	++++	++++	++++	++++	++++
0.04	++++	++++	++++	++++	++++	++++	++++	++++	++++
0.06	++++	++++	++++	++++	++++	++++	++++	++++	++++
0.08	++++	++++	++++	++++	++++	++++	++++	++++	++++
0.1	++++	++++	++++	++++	++++	++++	++++	++++	++++

TABLE 12

Results with the heated serum of a person on the thirteenth day of typhoid fever; agglutination titer 1: 40

DOSE OF SERUM	RESULTS WITH DIFFERENT ANTIGENS								
	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6	No. 7	No. 8	No. 9
cc.									
0.01	+++	−	−	−	−	−	−	−	+
0.02	++++	+	++	−	−	−	−	−	++
0.04	0	++	++++	−	−	−	−	−	++++
0.06	0	++++	++++	−	−	+	+	−	++++
0.08	++++	++++	++++	−	−	+++	++++	+++	++++
0.1	++++	++++	++++	−	−	+++	++++	+++	++++

TABLE 13

Results with the heated serum of a patient convalescent from typhoid fever; agglutination titer 1: 40

DOSE OF SERUM	RESULTS WITH DIFFERENT ANTIGENS								
	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6	No. 7	No. 8	No. 9
cc									
0.01	++++	−	+	−	−	−	±	−	++++
0.02	++++	−	++	−	−	−	+	+	++++
0.04	++++	−	+++	−	−	−	++	+++	++++
0.06	++++	+	+++	−	−	−	+++	+++	++++
0.08	++++	+	+++	−	−	−	++++	++++	++++
0.1	++++	++	+++	−	−	−	++++	++++	++++

TABLE 14

Results with the heated serum of a patient on the seventeenth day of typhoid fever; agglutination titer 1: 40

DOSE OF SERUM	RESULTS WITH DIFFERENT ANTIGENS								
	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6	No. 7	No. 8	No. 9
cc.									
0.01	++++	++++	++++	−	−	−	−	−	+
0.02	++++	++++	++++	−	−	+	+	+++	+++
0.04	++++	++++	++++	−	−	++++	++	++++	++++
0.06	++++	++++	++++	−	−	++++	+++	++++	++++
0.08	++++	++++	++++	−	−	++++	++++	++++	++++
0.1	++++	++++	++++	−	−	++++	++++	++++	++++

TABLE 15

Results with the heated serum of a patient convalescent from typhoid fever; agglutination titer 1: 40

DOSE OF SERUM	RESULTS WITH DIFFERENT ANTIGENS								
	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6	No. 7	No. 8	No. 9
cc.									
0.01	+++	++++	+	−	−	−	+	+	+++
0.02	++++	++++	++++	−	−	++	+++	+++	++++
0.04	++++	++++	++++	−	−	++++	++++	++++	++++
0.06	++++	++++	++++	−	−	++++	++++	++++	++++
0.08	++++	++++	++++	−	+	++++	++++	++++	++++
0.1	++++	++++	++++	−	+	++++	++++	++++	++++

TABLE 16

The results with the heated serum of a person who had typhoid fever one year ago; agglutination titer 1: 40

DOSE OF SERUM	RESULTS WITH DIFFERENT ANTIGENS							
	No. 1	No. 2	No. 3	No. 4	No˙ 6	No. 7	No. 8	No. 9
cc.								
0.01	+++	−	−	−	−	−	−	+++
0.02	+++	+	+++	−	−	+	++	+++
0.04	++++	++++	++++	+	++	++++	++++	++++
0.06	++++	++++	++++	++++	++	++++	++++	++++
0.08	++++	++++	++++	++++	+++	++++	++++	++++
0.1	++++	++++	++++	++++	+++	++++	++++	++++

TABLE 17

Results with the unheated serum of a person who had typhoid fever and typhoid vaccine four years ago; agglutination titer 1: 10

DOSE OF SERUM	RESULTS WITH DIFFERENT ANTIGENS								
	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6	No. 7	No. 8	No. 9
cc.									
0.01	++++	+	++++	++++	−	+	++++	++++	++
0.02	++++	+	++++	0	++++	+	++++	++++	++++
0.04	++++	++	++++	++++	++++	+++	++++	++++	++++
0.06	++++	++	++++	++++	++++	++	++++	++++	++++
0.08	++++	++	++++	++++	++++	+	+++	++++	++++
0.1	+++	+++	++++	++++	++++	+	+++	++++	++++

TABLE 18

Results with the unheated serum of a person who had vaccine two years ago; agglutination titer 1: 20

DOSE OF SERUM	RESULTS WITH DIFFERENT ANTIGENS								
	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6	No. 7	No. 8	No. 9
cc.									
0.01	++++	−	++++	++++	++++	+++	++++	++++	++++
0.02	++++	++++	++++	++++	++++	++++	++++	++++	++++
0.04	++++	++++	++++	++++	++++	++++	++++	++++	++++
0.06	++++	++++	++++	++++	++++	++++	++++	++++	++++
0.08	+++	+++	++++	++++	++++	+++	+++	+++	+++
0.1	+++	+++	++++	++++	++++	+++	+++	+++	+++

TABLE 19

Results with the unheated serum of a person who had typhoid fever eighteen years and vaccine six years ago; agglutination titer 1: 40

DOSE OF SERUM	RESULTS WITH DIFFERENT ANTIGENS								
	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6	No. 7	No. 8	No. 9
cc.									
0.01	++++	−	+	−	−	−	++++	++++	+++
0.02	++++	+	+++	+	++	−	++++	++++	++++
0.04	++++	++++	++++	++++	++++	−	++++	++++	++++
0.06	++++	++++	++++	++++	++++	++	++++	++++	++++
0.08	++++	++++	++++	++++	++++	−	++++	++++	++++
0.1	+++	++++	++++	++++	++++	−	++++	++++	++++

TABLE 20

Results with the unheated serum of a person who had vaccine one year ago; agglutination titer 1: 40

DOSE OF SERUM	RESULTS WITH DIFFERENT ANTIGENS								
	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6	No. 7	No. 8	No. 9
cc.									
0.01	++++	++++	++++	++++	++++	−	++++	++++	++++
0.02	++++	++++	++++	++++	++++	++	++++	++++	++++
0.04	++++	++++	++++	++++	++++	++++	++++	++++	++++
0.06	++++	++++	++++	++++	++++	++++	++++	++++	++++
0.08	++++	++++	++++	++++	++++	+	++++	++++	++++
0.1	++++	++++	++++	++++	++++	−	++++	++++	++++

TABLE 21

Results with the heated serum of a person who had vaccine two years ago; agglutination titer 1: 20

DOSE OF SERUM	RESULTS WITH DIFFERENT ANTIGENS							
	No. 1	No. 2	No. 3	No. 4	No. 6	No. 7	No. 8	No. 9
cc.								
0.01	−	−	−	+	−	−	−	−
0.02	−	−	−	+	−	−	+	−
0.04	−	−	−	++	−	−	+	−
0.06	−	+	−	++	−	+	++	±
0.08	−	+	−	++	−	+	++	+
0.1	−	+	−	+++	−	+	+++	++

TABLE 22

Results with the heated serum of a person during immunization with typhoid vaccine; agglutination titer 1: 160

DOSE OF SERUM	RESULTS WITH DIFFERENT ANTIGENS								
	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6	No. 7	No. 8	No. 9
cc.									
0.01	−	−	−	−	−	−	−	−	−
0.02	−	−	−	−	−	−	−	−	−
0.04	++	−	−	−	−	−	+	−	++
0.06	++++	+	−	−	−	−	++	+	++++
0.08	++++	++	−	−	−	−	++	+	++++
0.1	++++	+++	−	−	−	−	++	+	++++

TABLE 23

Results with the heated serum of a person who had vaccine one year ago; agglutination titer 1: 20

DOSE OF SERUM	RESULTS WITH DIFFERENT ANTIGENS							
	No. 1	No. 2	No. 3	No. 4	No. 6	No. 7	No. 8	No. 9
cc.								
0.01	−	−	−	−	−	−	−	−
0.02	−	−	−	−	−	−	−	−
0.04	−	−	−	−	−	−	−	−
0.06	−	−	−	−	−	−	−	−
0.08	−	−	−	−	−	−	−	−
0.1	−	−	−	−	−	−	−	−

TABLE 24

Results with the heated serum of a person who had vaccine about two years ago; agglutination titer 1: 80

DOSE OF SERUM	RESULTS WITH DIFFERENT ANTIGENS								
	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6	Nc. 7	No. 8	No. 9
cc.									
0.01	−	−	−	−	−	−	−	−	−
0.02	−	−	−	−	−	−	−	−	−
0.04	−	−	−	−	−	−	−	−	−
0.06	−	−	−	−	−	−	−	−	−
0.08	+	−	−	−	−	−	−	−	−
0.1	++	−	−	−	−	−	−	−	−

TABLE 25

Results with the heated serum of a person who had typhoid fever eighteen years ago and vaccine two years ago; agglutination titer 1: 40

DOSE OF SERUM	RESULTS WITH DIFFERENT ANTIGENS							
	No. 1	No. 2	No. 3	No. 4	No. 6	No. 7	No. 8	No. 9
cc.								
0.01	−	−	−	−	−	−	−	−
0.02	−	−	−	−	−	−	−	−
0.04	−	−	−	−	−	−	−	−
0.06	−	−	−	−	−	−	−	−
0.08	−	−	−	−	−	−	−	−
0.1	−	−	−	−	−	−	−	−

TABLE 26

Results with the heated serum of a person who had vaccine four years ago; agglutination titer 1: 10

DOSE OF SERUM	RESULTS WITH DIFFERENT ANTIGENS							
	No. 1	No. 2	No. 3	No. 4	No. 6	No. 7	No. 8	No. 9
cc.								
0.01	−	−	−	−	−	−	−	−
0.02	−	−	−	−	−	−	−	−
0.04	−	−	−	−	−	−	−	−
0.06	−	−	−	−	−	−	−	−
0.08	−	+	−	−	−	−	−	−
0.1	−	+	−	−	−	−	+	0

TABLE 27

Results with the heated serum of a person never having had typhoid fever or vaccine; agglutination test showed negative result

DOSE OF SERUM	RESULTS WITH DIFFERENT ANTIGENS							
	No. 1	No. 2	No. 3	No. 4	No. 6	No. 7	No. 8	No. 9
cc.								
0.01	–	–	–	–	–	–	–	–
0.02	–	–	–	–	–	–	–	–
0.04	–	–	–	–	–	–	–	–
0.06	–	–	–	–	–	–	–	–
0.08	–	–	–	–	–	–	–	–
0.1	–	–	–	–	–	–	–	–

showed that all kept uniformly well and none acquired markedly increased anticomplementary activities.

b. Hemolytic properties of the different antigens. The antigens varied considerably in hemolytic activity, as indicated in tables 1; antigens 3, 4 and 6 were markedly hemolytic and especially antigen 6, which was a suspension of ground dried bacilli in saline solution. Antigen 3 was a fourteen-day broth culture heated at 60°C. and preserved with 0.5 per cent phenol; antigen 4 was the filtrate of a heated suspension in distilled water, prepared after the method commonly employed for the preparation of gonococcus antigen.

The remaining antigens 1, 2, 5, 7, 8 and 9 were quite free of hemolytic substances.

c. Antigenic properties of the different antigens. As previously stated these were determined in a series of comparative tests in which each antigen was employed in an amount equal to one-third its anticomplementary unit with increasing amounts of human and rabbit immune sera; all antigens were tested at one time with each serum and with the same hemolytic system in order to render the tests strictly comparative.

The results observed with four rabbit immune sera of varying agglutinin content, are shown in tables 3, 4, 5, 6, 7 and 8; chart 1 shows graphically the variation in antigenic sensitiveness of the different preparations. Tables 9 and 10 also summarize the results, giving the smallest amount of each immune serum heated and unheated, yielding reactions with the respective antigens (chart 1).

Tables 10 to 26 give the results observed with the various antigens and the sera of persons with typhoid fever or convalescent from this infection; also the sera of persons who had typhoid fever or typhoid-paratyphoid vaccine at varying periods prior to the time when sera were collected for these tests.[1] Table 27 gives the results observed with a normal serum.

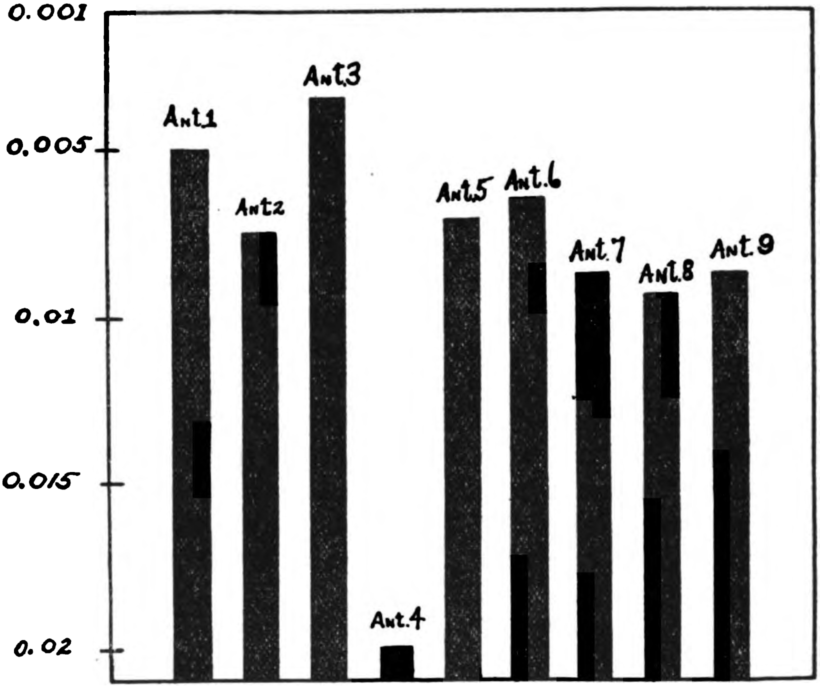

CHART 1. SHOWING THE COMPARATIVE ANTIGENIC SENSITIVENESS OF THE VARIOUS ANTIGENS WITH RABBIT IMMUNE SERA

As shown in these tables the antigens varied considerably in antigenic sensitiveness and may be summarized somewhat as follows in order of delicacy and sensitiveness.

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[1] I am indebted to Dr. John Eiman and Dr. Stanley P. Reiman for several of these sera from typhoid fever patients.

1. Antigen 3 was uniformly most antigenic in all tests; this was one of the simplest, being a fourteen-day broth culture killed by heating at 60°C. for one hour and preserved with 0.5 per cent phenol.

2. Antigen 1 was generally second in antigenic value and was prepared by suspending living bacilli removed from agar slant culture in sterile saline solution as required.

3. Antigens 2, 6, 7, 8 and 9 were about equal in antigenic activity and generally represented antigens prepared by grinding dried bacillary sediment secured with or without alcoholic precipitation, and suspending the very fine powder in saline solution.

4. Antigen 4 was uniformly poorest in antigenic sensitiveness and this result is of considerable significance inasmuch as it was prepared after a method commonly employed in the preparation of gonococcus antigen.

In general terms the results of this study with antigens of *B. typhosus* have shown that the best products are those in which are used whole bacilli in suspension with or without their soluble products elaborated during growth in fluid culture media; the next best are those antigens composed of thoroughly disrupted bacillary bodies in suspension and the lowest in antigenic value was the antigen prepared by autolysing the bacilli for intracellular substances and utilizing the filtrate which probably carries the soluble products and lacks the insoluble bacillary bodies. Exactly similar results and conclusions have been recorded by Kolmer and Brown in a study of gonococcus antigens, previously referred to.

A further result of this study was to show the marked effect of heating upon the typhoid antibody concerned in complement-fixation with human and to lesser extent with rabbit sera; all of the unheated sera of persons with typhoid fever or convalescing therefrom and the majority of those who had received typhoid-paratyphoid vaccine, yielded a positive reaction with the majority of antigens; after heating at 56°C. for thirty minutes a marked reduction in the degree of complement-fixation was observed; this was due, presumably, in part to the thermolability of the antibody inasmuch as the unheated sera were used fresh and found free of demonstrable anticomplementary activity in the serum control tubes, which always showed complete hemolysis.

CONCLUSIONS

1. Nine antigens prepared of a single strain of *B. typhosus* after various methods have shown well defined differences in antigenic sensitiveness.

2. Antigens prepared from living or dead suspensions of bacilli in saline solution or culture broth proved most antigenic; antigens prepared by suspending the powder of dried and ground bacilli in saline solution proved next best in antigenic sensitiveness and an antigen prepared of the filtrate of bacilli autolysed in distilled water aided by heating at a high temperature, proved least antigenic. The method of preparing typhoid antigen has, therefore, a marked effect upon the occurrence and degree of complement-fixation tests and the same is probably true of bacterial antigens in general.

3. The anticomplementary activity of the various antigens did not appear to differ to a marked extent; also, several were more hemolytic than the others; all of the antigens appeared to keep uniformly well over a period of six weeks at or near the freezing point.

4. The general result of this study and a review of investigations by others indicates that the similar bacterial antigens in which is employed the whole microorganism either living or dead in physiological saline solution or in culture broth, are superior to filtrates and constitute the antigens of choice for the conduct of complement-fixation tests in bacterial infections. I beg to express my appreciation to Professor Kolner for directions and aid in conducting this work.

REFERENCES

(1) Kolmer, J. A., and Brown, C. P. Complement fixation in gonococcus infections. Jour. Infect. Dis., 1914, 15, 6–21.

(2) Kolmer, J. A., and Berge, J. H. The relation of the typhoid in skin reaction to immunity in typhoid fever. Jour. Immunology, 1916, 1, 409.

(3) Kolmer, J. A. The relation of the diphtheria skin reaction to immunity in diphtheria. Jour. Immunology, 1916, **1**, 443.

(4) Kolmer, J. A., Matsunami, T., and Harkins, M. J. The relation of the bronchisepticin skin reaction to immunity in canine distemper including the bactericidal action of dog serum for B. brochisepticus. Jour. Immunology, 1916, **1**, 571.

(5) Hitchens, A. P., and Hansen, G. Studies on antibacterial serums. A stable bacterial antigens with special reference to meningococci. Jour. Immunology, 1916, **1**, 355.

(6) Small, J. C. A method of preparing bacterial antigens. Jour. Immunology, 1918, **3**, 413.

(7) Wilson, M. A. A contribution to the study of the complement fixation reaction in tuberculosis. Jour. Immunology, 1918, **3**, 345.

(8) Kolmer, J. A., and Trist, M. S. Non-specific complement fixation by normal rabbit serum. Jour. Infect. Dis., 1916, **18**, 20–26.

AN EXPERIMENTAL STUDY OF THE EFFECT OF AUTOGENOUS B. COLI VACCINES ON THE INTESTINAL COLON BACILLI OF DOGS

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From time to time, within recent years, the use of autogenous *B. coli* vaccines has been advocated as a therapeutic measure in the treatment of such conditions as chronic intestinal toxaemia (1) and eczema (2) on the assumption that the toxic substances giving rise to these conditions are produced through the activities of certain *B. coli* vegetating in the intestinal tract, and that these strains may be suppressed or eliminated through specific immunization. Apparently, however, this mode of treatment has not been substantiated by any experimental evidence either that the *B. coli* of the intestinal tract may be controlled through specific therapy or, if particular strains are reduced in numbers through this procedure, that the effect obtained is more than transitory. The study reported here was undertaken with the hope of throwing some light upon these points.

Normal dogs were utilized in these experiments in which an attempt was made to reduce in numbers or eliminate certain strains of *B. coli* naturally vegetating in their intestines.

All organisms belonging in the colon group ferment lactose, but only certain varieties split sucrose. Advantage was taken of these distinctions within the group in selecting strains for the preparation of the vaccine and in estimating the specific effect of inoculation on the distribution of colon types in the fecal specimens. In other words, if there exists any rational basis for attempting to control in a practical way through specific vaccine therapy the types of *B. coli* within the intestinal tract, then in these experiments inoculation with representatives of the sucrose-positive *B. coli* should cause an elimination or, at least, a marked reduction of these types as revealed in examinations of the fecal specimens.

The dogs, used in these experiments, were kept on a constant diet of boiled rice and boiled beef hearts in the ratio by weight of about 2 to 1. This diet was found by one of us (3) to be favorable for the development of an intestinal flora dominated by *B. coli*. In fact often the only colonies appearing on the Endo plates were *B. coli*-like. The amount of rice and meat fed was not weighed each day, as there would be no advantage in such precautions, but the relative proportions were kept approximately constant.

The fecal specimens were natural movements collected in the morning. Rather heavy emulsions, representative of the whole stool, were made in normal saline solution, and from suitable dilutions Endo plates were seeded. In the preparation of these Endo plates sucrose was substituted for the usual lactose. On these plates, of course, the sucrose fermenting strains of *B. coli* appeared as red colonies, whereas the varieties of *B. coli* which cannot split sucrose gave rise to white colonies. After twenty-four or more hours incubation the ratio of "whites" to "reds" among the *B. coli*-like colonies was determined and recorded. Frequent control tests were made to demonstrate the sucrose fermenting properties of the bacilli forming the red colonies and they were invariably found capable of splitting this sugar. Differential cultural tests were also carried out on a large number of isolated strains to establish their identity. Among the several hundred cultures examined no representative of the *B. aerogenes* type was encountered. Of one hundred sucrose-positive, gelatin-negative cultures from one dog thirty were positive for salicin and seventy were negative. According to Levine's (4) classification of the sucrose fermenters the salicin-positive types should be designated as *B. neapolitanus* and the salicin-negative as *B. communior* or *B. coscoroba* depending upon motility. Among three out of the four dogs differential fermentation tests with the sucrose-positive *B. coli* showed the salicin-negative types considerably in the majority. Gelatin liquefying strains among the sucrose-positive cultures seemed to be comparatively rare as out of 150 strains examined only 10 liquefied gelatin; accordingly about 7 per cent of these cultures should be placed in the *B. cloacae* group. The main objective in these differential cultural tests was, of course, the selection of sucrose-positive *B. coli* representative of all present in the intestinal flora. All the varieties of sucrose-positive *B. coli* isolated were incorporated in the vaccine except the gelatin liquefiers.

For the purpose of these experiments, then, a definite group of *B. coli*, viz., the sucrose fermenters, was selected to test the practicability of controlling these and related intestinal organisms through specific immunization. These bacilli are constant and normal inhabitants of the intestinal tract and ones which may be recognized readily through differential cultural and serological tests. In some ways it would have been preferable if bacterial species foreign to the intestinal tract could have been utilized. Implantation of such foreign strains, however, does not seem to be possible, and no experiments along that line were attempted.

Preliminary examinations for each animal were made at regular intervals for a period of four to six weeks with the purpose of determining not only the average ratio and the degree of variation in the comparative prevalence of the red and white colon colonies for the normal animal, but also for the selection of representatives of the main cultural variants among the sucrose-positive *B. coli.* With these selected cultures a vaccine was prepared and also rabbits were immunized for the production of a specific anti-serum. When the range in the ratio of the red and white colonies for the normal animal had been determined, a series of vaccine inoculations were given and the cultural examinations were continued at frequent intervals. At each plating during the period of immunization ten well isolated red colonies were transferred to agar slants and agglutination tests were carried out with each, by using the serum from the rabbit inoculated with the vaccine cultures. This anti-serum had a titer of 1-5000 to 1-10,000 for each of the vaccine cultures.

Tests with the isolated strains were made macroscopically at dilutions of 1-50 and 1-500. A sucrose-positive strain was not considered serologically related to the vaccine cultures unless it was definitely agglutinated at the 1-500 dilution. The object of the agglutination tests was to determine whether the sucrose-positive *B. coli* persisting in the intestinal tract in spite of the vaccine treatments were serologically related to the strains used in the vaccine or were entirely distinct strains which had grown up and replaced the originally prominent sucrose-positive *B. coli* types.

As has already been intimated, both the salicin-positive and the salicin-negative representatives of the sucrose-positive *B. coli* were selected in preparing the autogenous vaccine. Agar slant cultures of these strains were emulsified in normal saline and killed by exposure at 60°C. for one hour; the count was determined by the Wright method. The dog inoculations were given subcutaneously generally every two or three days for three or four treatments and then after an interval of a week or more the series of injections were repeated. The dosage in general ranged from one billion to eight billion. Except for a fairly marked loss of weight the animals seemed to suffer no ill effect from the inoculations.

In connection with each of the four experimental animals evidence was produced that treatment with an autogenous *B. coli* vaccine tended to cause a reduction in numbers and, in some instances, an apparently almost complete elimination of the particular varieties of *B. coli* used for immunization. This elimination, however, was more apparent than real; for subsequent examinations would reveal the presence of the same colon types, although it might be in very small numbers. In fact there was no evidence that it was possible, even with very large dosage of antigen to cause their disappearance permanently and completely.

In the tabulation the results for each dog is given. As may be noted, a considerable individual variation was encountered in the readiness and degree to which the animals reacted to the vaccine. Dogs 2 and 3 responded quite readily, whereas numbers 1 and 4 were more refractory. With dog 1 a preliminary examination period of five weeks revealed, on the average, an approximately equal number of red (sucrose-positive) and white (sucrose-negative) *B. coli* colonies. Following a billion dosage of the autogenous vaccine, repeated four times, the relative number of red colonies decreased until a week after the last injection they were outnumbered by the white colonies, 9 to 1. Soon, however, although an increased dosage was given, a change occurred in the ratio which would have been misleading unless agglutination tests had been carried out simultaneously. Apparently the vaccine had lost its effectiveness in that the white *B. coli* colonies were outnumbered by the red on the average 1 to 2. That this inference had no basis in fact was revealed in an examination of the agglutination results. For, whereas, in the previous period 91 per cent of the red colonies surviving the vaccine treatment yielded positive agglutinations at 1-500 dilution of the anti-serum, now an average of only 26 per cent agglutinated positively; indicating that serologically unrelated strains of sucrose-positive *B. coli* had grown up and replaced the original varieties with which the vaccine was prepared. Further examinations indicated that this substitution was only temporary as at the end of the period of examinations of this dog 86 per cent of the sucrose-positive *B. coli* agglutinated positively with the specific anti-serum, although on the average the white colonies now outnumbered the red five to one. This tendency of serologically unrelated strains of sucrose-positive *B. coli* to grow up was noted also with dogs 2 and 3, appearing in each instance at about the same stage of the immunization process, but in no case could these substituting strains maintain themselves.

In experiments of this character it is important to carry out a long series of preliminary observations to determine the degree to which the types of *B. coli* selected vary in numbers under ordinary conditions. A period of six to eight weeks was considered adequate for this purpose. After inoculations were started about two to three weeks elapsed before definite results were noted in the differential *B. coli* determinations. In dog 3 an apparent rapid elimination of sucrose-positive strains occurred within a week after the first treatment with the vaccine. It is questionable, however, if this change may be ascribed to the specific action of the vaccine. As regards dosage it was found than an inoculation with about 2 to 4 billion *B. coli* secured the maximum effect, and that nothing was gained by increasing the amount above this point. In one experiment a dosage as small as 100 million was employed. After five inoculations covering a period of three weeks there seemed to be some slight effect, but not as definite as following the larger dosage.

Observations were not continued long enough to determine definitely how enduring the effect of the inoculations might be. In the case of one animal, dog 2, the effect was undiminished after the lapse of ten weeks following the last of a series of six inoculations. With dog 3 the effect seemed to have largely worn off in seventeen weeks, but during the interval the diet had been somewhat changed. Diet, in fact, is the factor of prime importance in the determination of the types of bacteria pullulating in the intestinal tract, and without an uniform diet the results of these experiments would have been indefinite and misleading. An experiment with dog 4 showed how readily modification of the diet would upset the relative distribution of *B. coli* types established through vaccine inoculations. A week after the animal had completed the immunization process and the sucrose-negative *B. coli* had become predominant, fifty grams of lactose was added to the diet of rice and meat. Within three days there was noted a very marked increase in the *B. coli* count with about an equal number of red and white *B. coli* colonies on the Endo plates. This apparent inhibition of the specific repressive action of *B. coli* inoculations continued as long as lactose was a part of the diet.

Although the question of the relative effect of autogenous and heterogenous vaccines was not made a feature of this study, yet some experiments with dog 2 indicated that to obtain marked results it was necessary to use an autogenous vaccine. After a preliminary observation period of six weeks, this animal was given three weekly inoculations of the *B. coli* vaccine prepared from cultures derived from dog 1 in a dosage of two billion. Very little in the way of definite effect, however, was noted until an autogenous vaccine was used when a marked and persistent decrease in the relative numbers of the sucrose-positive *B. coli* followed.

With one animal (dog 4) an attempt was made to correlate the cultural results with the rise of antibody (agglutinin) in the blood following the inoculations. The serum from this animal before inoculation agglutinated an autogenous sucrose-positive *B. coli* culture at 1-160 dilution. This amount of agglutinin showed first a definite enhancement about two weeks after a series of three inoculations had been given, when the titer rose to 1-640. Shortly after this increase in anti-body content of the blood, a definite decrease occurred in the number of sucrose-positive *B. coli* in the fecal specimens examined. Continued inoculations with larger doses of the vaccine finally sent the titer up to 1-1280, with a coincident repression of the sucrose-positive *B. coli* as shown in the tabulation. A number of agglutination tests with extracts of the fecal matter were carried out to determine to what extent this antibody found its way into the intestinal tract. Positive results were obtained with watery defecations and especially with saline extracts of the mucus, but not with extracts from emulsions of the formed or semi-formed matter.

These experiments with dogs have established the fact that it is possible through the use of autogenous vaccines to effect at least a temporary suppression of corresponding strains of *B. coli* naturally vegetating in the intestinal tract. Assuming that some varieties of *B. coli* may be of importance in connection with intestinal toxaemia, a certain amount of experimental justification is accorded the employment of an autogenous vaccine therapy. In fact it should be much easier to effect by this means the suppression of parasitized *B. coli* than of the normal vegetative types which are firmly established in the intestinal tract. Such being the case one of the real difficulties in applying this form of therapy is to determine just what strains of *B. coli* may be giving rise to toxic products and to incorporate these in

TABLE 1

Effect of inoculation with B. coli vaccine on the types of B. coli vegetating within the intestines of dogs

DOG	DATES OF EXAMINATIONS	DATES AND DOSAGE OF VACCINATION	AVERAGE RATIO OF SUCROSE-NEGATIVE TO SUCROSE-POSITIVE B. COLI	PER CENT OF SUCROSE-POSITIVE B. COLI AGGLUTINATING WITH ANTI-SERUM TO THE VACCINE CULTURES	REMARKS
1	XI-12, 19; XII-10, 12, 16, 20, 1918		1-1		Pre-vaccination period
1	XII-28, 31, 1918; 1, 3, 4, 8, 1919	XII-21, 1 B. XII-28, 1 B. XII-30, 1 B. I- 2, 1 B.	3-1		Vaccination period
1	I-14, 20, 22, 1919	I- 9, 1 B. I-11, 1 B. I-13, 1 B.	9-1	91	
1	I-24, 27, 30; II-2, 4, 6, 1919	I-24, 3 B. I-28, 3 B. I-30, 3 B.	1-2	26	Increase in number of other strains of sucrose-positive B. coli
1	II-9, 11, 13, 15, 17, 1919		2⅓-1	58	
1	II-25, 27; III-3, 5, 7, 1919	II-20, 4 B. II-26, 4 B.	5-1	86	Above strains have decreased in numbers
2	I-9, 14, 16, 20, 22, 24, 27, 30, 1919		1-3	94	Pre-vaccination period
2	II-2, 4, 6, 9, 11, 13, 17, 19, 1919		2-1	81	Pre-vaccination period
2	II-25, 27; III-3, 5, 7, 12, 1919	II-20, 2 B., Dog I vaccine II-26, 2 B., same III-5, 2 B., same	2-1	91	Heterogenous vaccine

2	III-15, 18, 21, 26, 1919	III-13, 2 B., Dog II vaccine III-19, 4 B., same III-24, 4 B., same	1-3	39	Autogenous vaccine
2	III-28; IV-1, 3, 7, 9, 1919		6-1	28	
2	IV-11, 14, 1919		All sucrose-negative		Apparent elimination of sucrose-positive B. coli
2	IV-17, 21, 23, 25; V-2, 5, 8, 12, 1919		4-1	71	
2	V-15, 1919		All sucrose-negative		
2	V-19, 21, 23, 26, 28, 31, 1919		18-1	17	
2	VI-4, 6, 9, 1919		12-1	83	
2	VI-11, 13, 16, 18, 1919	VI-10, 8 B. VI-16, 8 B. VI-18, 8 B.	13-1	80 (2 tests)	Large dosage of vaccine
2	VI-20, 23, 27, 30 1919		7-1	13 (3 tests)	
3	III-12, 14, 1919		12-1	100	Pre-vaccination period
3	III-18, 1919		All sucrose-positive		
3	III-21, 26, 28; IV-1, 1919		11-1	90	
3	IV-3, 1919		All sucrose-positive		
3	IV-7, 9, 11, 17; 21, 23, 25, 1919		5-1	70	
3	IV-29; V-2, 5, 8, 1919		2-1	95	
3	V-12, 1919	V-9, 2 B.	90-1	0	Vaccination period. Autogenous vaccine
3	V-15, 1919		All sucrose-negative		
3	V-19, 21, 23, 26; 28, 31, 1919	V-20, 2 B. V-27, 4 B.	6-1	45	

DOG	DATES OF EXAMINATIONS	DATES AND DOSAGE OF VACCINATION	AVERAGE RATIO OF SUCROSE-NEGATIVE TO SUCROSE-POSITIVE B. COLI	PER CENT OF SUCROSE-POSITIVE B. COLI AGGLUTINATING WITH ANTI-SERUM TO THE VACCINE CULTURES	REMARKS
3	VI-3, 1919		All sucrose-negative		
3	VI-4, 6, 9, 11, 13, 1919	VI-6, 4 B.	16-1	64	
3	VI-16, 18, 20, 23, 25, 27, 30; VII-2, 1919		31-1	32 (average of 5 examinations)	
3	VII-5, 1919		All sucrose-negative		
3	VII-7, 9, 11, 1919		25-1	30	
4	VIII-12, 14, 21, 26, 29; IX-3, 8, 12, 1919		17-1		Pre-vaccination period
4	IX-18, 22, 29; X-3, 6, 9, 1919		5-1		
4	X-13, 15, 16, 21, 23, 25, 1919	X-14, 3 B. X-17, 3 B. X-20, 3 B.	1-3		Vaccination period. Autogenous vaccine
4	X-27, 29, 31; XI-3, 5, 1919	X-27, 6 B. XI- 1, 6 B.	44-1		
4	XI-7, 10, 13, 14, 17, 1919	XI- 8, 6 B. XI-15, 6 B.	28-1		
4	XI-24, 26, 28; XII-2, 4, 1919		2-1		Lactose (50 grams) added to diet November 18, and after

the vaccine. Apparently heretofore vaccines of this character have been made up in a rather hit or miss fashion. It should also be borne in mind that diet is the most important factor in the regulation of the intestinal flora. In fact these experiments indicate that an attempt to control types of bacteria germinating in the intestinal tract through specific vaccine therapy is futile unless the diet is also carefully regulated.

CONCLUSIONS

1. In the case of a number of dogs it has been found possible to effect the temporary suppression of a certain variety of *B. coli* normal to the intestinal tract through inoculations with a specific vaccine.
2. Autogenous vaccines are apparently necessary for marked results and the dosage must be large.
3. Cultural results indicating a decrease in numbers of the type of *B. coli* in question were associated with a coincident rise of specific anti-body in the blood.
4. A uniform diet must be maintained, otherwise the effect of the vaccine will be obscured.

REFERENCES

(1) **Satterlee**, G. R.: Journ. Amer. Med. Assoc., 1916, **67**, 1729.
(2) **Jackson and Pichard**: Calif. State Journ. of Med., 1918, **16**, No. 7.
(3) **Torrey**, J. C.: Journ. Med. Research, 1919, **39**, 415.
(4) **Levine, Max**: Journ. of Bact., 1916, **1**, 619.

EXPERIMENTAL STUDY OF THE SENSITIZED CHOLERA ANTIGEN

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Knowing that ordinary heated vaccine has no complete prophylactic action upon experimental typhoid fever, Besredka (1), in 1902, first used the so-called sensitized vaccine, which was made by combining the living typhoid bacilli with the useful constituents of immune serum. Later he investigated with Metchnikoff (2) its prophylactic value against experimental typhoid fever, and they found it effective and not dangerous to the human body. In 1914 Ichikawa, in Japan, experimented with the non-heated sero-vaccine upon typhoid fever patients with good results. In 1916, when the outbreak of cholera was at its height in Japan, the cholera sensitized vaccine was prepared by Shiga and his associates (3) and favorable results were obtained. Later Yabe published an article on cholera vaccine, its weak reaction and rapid development of antibodies, as compared with the ordinary vaccine.

We regret, however, that the sensitized vaccine in solution is not stable, and thus it cannot be kept for a long time in soluble form. In order to keep the sensitized vaccine without losing its power, as long as possible, it has been made in powder form by drying it in a vacuum desiccator over calcium chloride. We found that the sensitized cholera vaccine powder thus prepared has nearly the same effect as the bacilli suspension in liquid form, even after a period of nineteen months. We shall describe the experimental results in the following manner:

I. METHOD OF PREPARING CHOLERA VACCINE

a. Preparation of sensitized cholera vaccine

The cholera bacilli from the agar culture were incubated for eighteen hours at 37°C. and weighed. Then were added together 18 cc. of saline solution and 2 cc. of cholera immune serum of a rabbit (the bacteriological unit of which was more than 0.0001) for each gram of the above. After complete combination took place between the bacilli and the immune bodies, it was incubated at 37°C. for about two hours, and then centrifugalized. The supernatant liquid was decanted and the sediment was washed twice with saline solution. Then as much saline solution was added, containing 0.5 per cent carbolic acid as was necessary to make 1 cc. contain 2 mgm. of bacilli.

b. Preparation of the sensitized cholera vaccine powder

The bacilli suspension thus prepared was taken upon a watch glass which was placed in the middle of the desiccator provided with calcium chloride. Then the air in the jar was pumped out by means of an air pump connected with it, and the jar was allowed to stand for about forty-eight hours at room temperature. When the vaccine became a brownish powder, it was kept in an ampule or tightly corked bottle.

The sensitized cholera vaccine powder thus made corresponds to 10 mgm. of living bacilli in 1 mgm. Before use, 0.2 mgm. vaccine powder is suspended in 1 cc. saline solution containing 0.5 per cent of carbolic acid, and the suspension is well shaken.

II. ANIMAL EXPERIMENTS

The animal experiments with the ordinary sensitized cholera vaccine have been published by Shiga and his associates as described above. We attempted to get the same results with the sensitized cholera vaccine powder nineteen months old, and to discover the difference in value, if any, between them.

Ten healthy rabbits between 2000 and 2500 grams body weight were divided into two groups, In the first group 1 cc.

(containing 2 mgm. of bacilli) of ordinary sensitized vaccine and in the second group 0.1 gram of the sensitized cholera vaccine powder (containing 2 mgm. of bacilli) was injected into the vein of the ear of each rabbit. On the ninth day following the injection, when the production of antibodies is always highest, the blood was withdrawn from the heart. The separated serum was heated for thirty minutes at 56°C. to be made inactive and then the following experiments were made with it.

1. Experiments on the development of agglutinin and bacteriolysin

In order to know proportionately how much of the antibody is agglutinin and bacteriolysin, and how much is present in this serum, the agglutination reaction and Neisser-Wechsberg's method were used. Results obtained are shown in table 1.

a. Experiment on agglutination index. One hundred test tubes were divided into two groups, the first group for the ordinary cholera sero-vaccine, and the second group for the powder vaccine, and then each group was arranged into five series of ten tubes each, each series for one immune rabbit. Into the first tube of each series was put 0.90 cc. of the saline solution and in the others 0.50 cc. and further in each first tube 0.10 cc. non-diluted immune serum of its rabbit. 0.5 cc. of this was removed and placed in the next test tube, and the process repeated for each succeeding tube. Then to each tube was added 0.50 cc. of living cholera bacilli in suspension (1 cc. of this suspension contains 1.0 mgm. agar culture of cholera bacilli in eighteen hours cultivation). The total volume in each tube was, thus, 1 cc. The mixtures were thoroughly shaken. Thereupon these tubes were incubated for two hours at 37°C., and the result was read as shown in table 1.

b. Neisser-Wechsberg's experiment. The serum was diluted as in the preceding experiment and 0.30 cc. of complement (1:10) and 0.50 cc. of bacilli suspension (1/500 mgm. in 1 cc.) were added to each dilution of serum. Then, after these tubes were incubated at 37°C. for two hours the agar medium was poured into each tube. When the agar was completely coagulated it

TABLE 1

RESULT IN BACTERIOLYTIC INDEX	GROUP I					GROUP II					CONTROL
	Number of rabbits										
	1	2	3	4	5	6	7	8	9	10	
	Agglutination index										
	1280	2560	1280	1280	5120	640	1280	1280	2560	1280	
0.05	+++	+++	++	++	+++	++	+++	++	+++	++	+++
0.025	++	+++	+	+	++	++	++	+	+++	+	+++
0.01	+	++	+	+	+	−	+	−	++	+	+++
0.005	−	+	−	−	+	−	−	−	+	−	+++
0.0025	−	+	−	−	−	−	−	−	−	−	+++
0.001	−	−	−	−	−	−	−	−	−	−	+++
0.0005	−	−	−	−	−	−	−	−	−	−	+++
0.00025	−	−	−	−	−	+	−	−	−	−	+++
0.0001	−	−	+	−	−	+	−	+	−	−	+++
0.00005	+	−	+	+	−	+	+	+	−	+	+++
0.000025	+	+	++	++	−	++	+	++	+	+	+++
0.00001	++	+	++	++	+	+++	++	+++	+	++	+++
0.000005	++	++	+++	+++	++	+++	+++	+++	++	++	+++
0.0000025	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
0.000001	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
0.0000005	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
0.00000025	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
0.0000001	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++

was incubated again for twenty-four hours and the number of colonies was counted.

According to table 1, the index of agglutination was 1280 to 5120 in rabbits of the first group and 640 to 2560 in those of the second group. The bacteriolytic result in the first group shows the index of 0.00025 to 0.000025 and in the second group of 0.0005 to 0.00005, thus proving that the agglutination and bacteriolytic indices are nearly the same for these two vaccines.

2. *Pfeiffer's experiment*

From the preceding experiments it was evident that the antibody was produced by the intravenous injection of 0.10 gram of this vaccine powder. In order to obtain a more accurate result, however, we tried the following experiment:

TABLE 2

DILUTION OF SERUM	GROUP I					GROUP II				
	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6	No. 7	No. 8	No. 9	No. 10
1:20	Alive	Alive	Alive	Died	Alive	Alive	Alive	Alive	Died	Alive
1:40	Alive	Alive	Alive	Alive	Alive	Alive	Alive	Died	Died	Alive
1:80	Alive	Alive	Alive	Alive	Died	Alive	Alive	Alive	Alive	Alive
1:160	Alive	Alive	Alive	Alive	Alive	Alive	Died	Alive	Alive	Died
1:320	Alive	Died	Alive	Alive	Alive	Alive	Alive	Alive	Alive	Died
1:640	Alive	Alive	Died	Alive	Alive	Died	Alive	Alive	Alive	Alive
1:1280	Died	Alive	Died	Died	Alive	Died	Died	Died	Alive	Died
1:2560	Died	Died	Died	Died	Alive	Died	Died	Died	Died	Died
1:5320	Died	Died	Died	Died	Died	Died	Died	Died	Died	Died
1:10640	Died	Died	Died	Died	Died	Died	Died	Died	Died	Died

One hundred healthy guinea-pigs from 150 to 200 grams body weight were divided into two groups. In the first group an immune serum of the common sensitized vaccine, and in the second group that of the sensitized vaccine powder, was injected intraperitoneally, then immediately 1 cc. of the living cholera bacilli suspension (that is twice the lethal dose) was introduced intraperitoneally into each of them, and examination made after twenty-four hours. The results are shown in table 2.

TABLE 3

DATE	GROUP I					GROUP II				
	Number of animal	Body weight	Dose of vaccine	Dose of living bacilli	Result	Number of animal	Body weight	Dose of vaccine	Dose of living bacilli	Result
day		*grams*	*cc.*	*mgm.*			*grams*	*cc.*	*mgm.*	
First	1	175	1	2	Died	61	200	1	2	Died
	2	181	1	2	Died	62	200	1	2	Died
	3	195	1	2	Died	63	200	1	2	Died
	4	190	1	2	Died	64	195	1	2	Died
	5	170	1	2	Died	65	198	1	2	Alive
	6	200		2	Died	66	200		2	Died
Second	7	170	1	2	Died	67	160	1	2	Died
	8	185	1	2	Died	68	182	1	2	Died
	9	190	1	2	Died	69	195	1	2	Died
	10	170	1	2	Died	70	199	1	2	Died
	11	192	1	2	Died	71	187	1	2	Died
	12	200		2	Died	72	198		2	Died
Third	13	170	1	2	Died	73	180	1	2	Died
	14	150	1	2	Died	74	160	1	2	Died
	15	172	1	2	Died	75	175	1	2	Died
	16	178	1	2	Died	76	165	1	2	Died
	17	198	1	2	Died	77	155	1	2	Died
	18	185		2	Died	78	200		2	Died
Fourth	19	185	1	2	Alive	79	195	1	2	Died
	20	195	1	2	Died	80	180	1	2	Died
	21	191	1	2	Died	81	180	1	2	Died
	22	192	1	2	Died	82	180	1	2	Alive
	23	155	1	2	Died	83	185	1	2	Died
	24	200		2	Died	84	200		2	Died
Fifth	25	190	1	2	Died	85	190	1	2	Alive
	26	195	1	2	Alive	86	155	1	2	Alive
	27	155	1	2	Died	87	175	1	2	Alive
	28	160	1	2	Alive	88	175	1	2	Died
	29	175	1	2	Alive	89	160	1	2	Died
	30	200		2	Died	90	200		2	Died
Sixth	31	190	1	4	Alive	91	190	1	4	Died
	32	170	1	4	Alive	92	195	1	4	Alive
	33	160	1	4	Died	93	180	1	4	Alive
	34	165	1	4	Died	94	170	1	4	Died
	35	155	1	4	Alive	95	170	1	4	Alive
	36	200		4	Died	96	195		4	Died

TABLE 3—*Concluded*

DATE	GROUP I					GROUP II				
	Number of animal	Body weight	Dose of vaccine	Dose of living bacilli	Result	Number of animal	Body weight	Dose of vaccine	Dose of living bacilli	Result
day		*grams*	*cc.*	*mgm.*			*grams*	*cc.*	*mgm.*	
Seventh	37	200	1	4	Alive	97	170	1	4	Alive
	38	195	1	4	Alive	98	170	1	4	Alive
	39	175	1	4	Alive	99	192	1	4	Alive
	40	180	1	4	Alive	100	190	1	4	Died
	41	195	1	4	Alive	101	185	1	4	Alive
	42	195		4	Died	102	200		4	Died
Eighth	43	190	1	4	Alive	103	200	1	4	Alive
	44	195	1	4	Alive	104	200	1	4	Alive
	45	175	1	4	Alive	105	200	1	4	Died
	46	165	1	4	Died	106	190	1	4	Alive
	47	165	1	4	Alive	107	190	1	4	Alive
	48	195		4	Died	108	200		4	Died
Ninth	49	160	1	4	Alive	109	170	1	4	Alive
	50	190	1	4	Alive	110	175	1	4	Died
	51	185	1	4	Died	111	159	1	4	Alive
	52	183	1	4	Alive	112	165	1	4	Alive
	53	180	1	4	Alive	113	177	1	4	Alive
	54	190		4	Died	114	200		4	Died
Tenth	55	180	1	4	Alive	115	155	1	4	Died
	56	182	1	4	Died	116	157	1	4	Died
	57	175	1	4	Died	117	168	1	4	Alive
	58	177	1	4	Alive	118	175	1	4	Alive
	59	180	1	4	Alive	119	177	1	4	Alive
	60	200		4	Died	120	200		4	Died

Comparing the results again with those of the above agglutination and bactericidal experiment, we have proved that the activities are nearly the same in both vaccines. Five control guinea-pigs used in this experiment all died within twenty-four hours.

3. Experiments on prophylaxis

We divided 100 healthy guinea-pigs into two groups; 1 cc. of the sensitized cholera vaccine was injected subcutaneously into each of the first group, and 0.10 cc. of the sensitized cholera vaccine powder into each of the second group. Then every day six healthy ones were selected from each group and 1 cc. of bacillary suspension (2 to 4 mgm. of bacilli) was injected intraperitoneally. Examination was made every twenty-four hours. As a control a healthy guinea-pig was taken every day and the same dose of the bacillary suspension was injected intraperitoneally, but all died within twenty-four hours.

In considering the result of the prophylactic experiment we found that the production of antibodies increases gradually from the fourth day, in each group, until it is at its highest on the seventh, eighth and ninth days, and begins to decrease again on the tenth day.

III. OBSERVATIONS ON THE REACTION IN THE HUMAN BODY, FOLLOWING THE INJECTION OF SENSITIZED CHOLERA VACCINE POWDER

The most important means for comparing the grade of the reaction is the recording of the body temperature after the injection and the local and general subjective symptoms.

The results observed after injection of the powdered preparation into the human body are shown in table 4.

There was almost no temperature reaction. In some cases we saw a slight induration, but no pain, with recovery in one or two days. The general symptoms were only slight lassitude or headache in a few patients but none were confined to bed. Here, also, we obtained a favorable result by using the sensitized vaccine powder, as in case of the common sensitized vaccine, in which the reaction is very slight.

We avoided injection into the aged, infants, invalids, drunkards and pregnant women and into cases with heart disease, pulmonary tuberculosis, nephritis and beri-beri.

TABLE 4

Reaction upon human body

NAME	SEX	AGE	CONSTITUTION	OCCUPATION	NUTRITION	DOSE OF INJECTION	TEMPERATURE BEFORE INJECTION	HIGHEST TEMPERATURE WITHIN 24 HOURS AFTER INJECTION	GENERAL SUBJECTIVE SYMPTOMS	LOCAL REACTION
		years				*cc.*	*degrees C.*	*degrees C.*		
S. S.	♀	26	Medium	Lodger in hospital	Good	1	36.1	36.2	Negative	Negative
T. C.	♀	22	Superior	Lodger in hospital	Very good	1	36.6	37.0	Negative	Slight tenderness
T. S.	♀	19	Medium	Lodger in hospital	Good	1	36.3	36.5	Lassitude	Negative
S. I.	♀	24	Medium	Lodger in hospital	Fair	1	36.1	36.0	Negative	Negative
Y. F.	♀	26	Superior	Lodger in hospital	Good	1	36.6	37.0	Lassitude	Feeling of swelling
K. T.	♀	20	Medium	Lodger in hospital	Good	1	36.4	36.0	Negative	Negative
T. I.	♀	20	Medium	Lodger in hospital	Very good	1	36.3	37.0	Slight heaviness in head	Tenderness
M. K.	♀	23	Medium	Lodger in hospital	Very good	1	36.9	37.0	Negative	Negative
M. K.	♀	26	Medium	Lodger in hospital	Very good	1	36.3	36.0	Negative	Negative
T. K.	♀	27	Superior	Lodger in hospital	Very good	1	36.4	36.5	Negative	Negative
S.	♂	36	Medium	Official	Fair	1	36.5	36.6	Negative	Negative
S.	♂	27	Superior	Official	Good	1	36.4	36.8	Lassitude	Slight pain
M.	♂	33	Superior	Doctor	Very good	1	36.5	36.6	Negative	Negative
S.	♀	42	Medium	Midwife	Good	1	36.5	36.3	Negative	Slight pain
S.	♀	18	Superior	Business woman	Good	1	36.4	36.4	Negative	Negative
O.	♂	33	Superior	Doctor	Good	1	36.2	36.9	Heaviness in head	Negative
H.	♂	35	Superior	Military officer	Very good	1	36.7	37.1	Heaviness in head	Feeling of swelling
S.	♂	18	Superior	Student	Very good	1	36.8	36.8	Negative	Negative
M.	♂	37	Medium	Policeman	Fair	1	36.4	37.0	Negative	Negative
I.	♂	33	Superior	Military officer	Good	1	36.6	36.5	Negative	Negative

CONCLUSIONS

1. The quantity of the agglutination and bacteriolysin produced by the treatment with the sensitized vaccine powder is nearly equal to that by the common sensitized vaccine.

2. The reaction on the part of the human body is very slight after the injection of either of these vaccines.

3. The sensitized vaccine powder does not decompose as rapidly as the common sensitized vaccine. It has been preserved for nineteen months without losing its value as an antigen.

REFERENCES

(1) Besredka, A.: Ann. Inst. Pasteur, 1902, **16**, 918.
(2) Metchnikoff, E., and Besredka, A.: Ann. Inst. Pasteur, 1911, **25**, 193.
(3) Shiga, K.: Kitasto's Arch. Exper. Med. 1918, **11**.

A DROPPING BOTTLE AS AN AID IN MACROSCOPIC SLIDE AGGLUTINATION

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The macroscopic slide agglutination method has been more or less generally known for years. The value of the method, however, has not been generally appreciated, probably due to the fact that most of the serums employed for identification purposes have not been sufficiently active to give an immediate or prompt agglutination even in low dilutions. Coca (1) utilized the method in cholera examinations and, to our knowledge, he was the first to publish a description of the method. We have utilized it for some years as a routine in examinations of feces for members of the typhoid-paratyphoid dysentery types (2) and also as an aid in the search for meningococcus carriers (3). As very potent sera become more generally available its use will undoubtedly spread to most laboratories.

There is one inconvenient feature in the method which we believe to have overcome by the device here reported. In placing on the slide the drops of diluted agglutinating serum and the saline or diluted normal serum for control it has been the custom to utilize the ordinary platinum loop. Where many colonies are to be tested this method is time-consuming and tedious.

The dropping bottle that we have devised to deliver the drops depends upon the use of a capillary delivery tube with a flat end and a rubber diaphragm to force the delivery of the drop.

The delivery tube is prepared from a piece of heavy walled tubing (thermometer type) having an outside diameter of about 4 mm. and a bore of about 1 mm. This is heated thoroughly in the flame and drawn out slowly. A piece is then cut as shown in the outline sketch (figure 1) and the larger end ground flat and smooth on a fine stone. The diameter of the bore is reduced to about the bore of 20 gauge hydpodermic needle.

The diaphragm is made from a so-called "no-air" stopper, size No. 2½. The cork end is cut off leaving only sufficient to give stability to the delivery tube. The delivery tube is thrust through the cork.

The bottle employed is the regulation ½ ounce wide mouth homeopathic vial. The neck of the bottle must be wider than the cork portion of the stopper to allow up and down play otherwise there will be no diaphragm action.

The bottles are partly filled with the diluted serums or saline and the drops are placed on the slide by holding the bottle vertical and pressing till the appropriate sized drop is delivered. With very little practice many drops can be delivered in a few seconds. The appropriate pressure to apply soon requires no conscious attention.

The amount of air or air tension in the bottle needs readjustment from time to time. Air can be introduced by inverting the bottle and pulling on the delivery tube. If there is too much air pressure this can be equalized by holding the bottle upright and pressing down the diaphragm which empties the tube and results in equalization of the pressure.

There are other possible applications of this dropping bottle. It may be utilized to deliver drops of saline or other fluids used in the preparation of smears of cultures. In the classroom it could be utilized to give out suspensions of microörganisms for smear examinations. Other similar uses will probably be found for it.

REFERENCES.

(1) Coca, Arthur F.: Bulletin Manila Med. Soc., 1910, **2**, no. 1.
(2) Krumwiede, Charles: Journ. Inf. Dis., 1918, **23**, 275; 1917, **21**, 141.
(3) Krumwiede, Charles: Journ. A. M. A., 1917, **6-9**, 358.

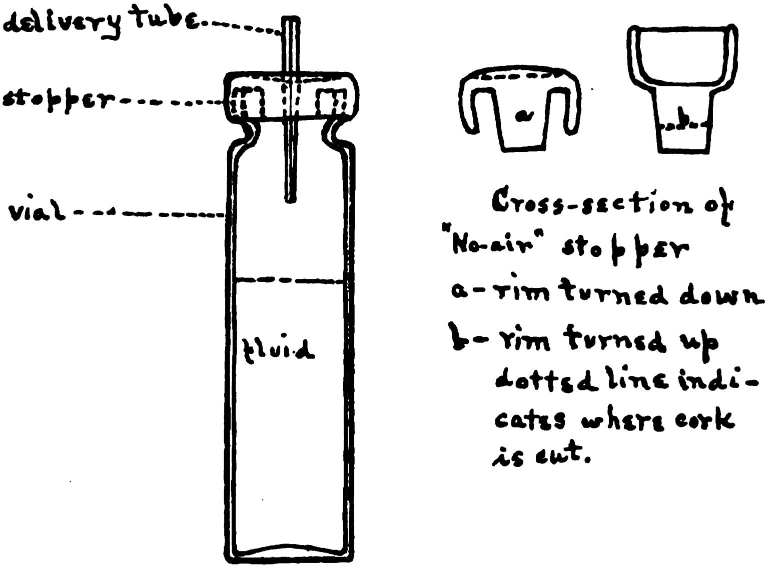
delivery tube
stopper
vial
fluid
a
b
Cross-section of
"No-air" stopper
a- rim turned down
b- rim turned up
dotted line indicates where cork
is cut.

FIG. 1.

THE COMPLEMENT FIXATION TEST FOR TUBERCULOSIS

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1. INTRODUCTION

In spite of the epoch-making discovery of Koch, the early diagnosis of tuberculosis is still a problem to be solved, even as it was one hundred years ago in the days of Laennec.

Although methods for the early diagnosis of tuberculosis are still wanted, the wide distribution of this disease is well established. In fact, tuberculosis, not unlike syphilis, must be constantly kept in mind in making a diagnosis, whatever may be the clinical picture of the case.

McCrae and Funk (1) in 1919 stated that, although the recognition of chronic pulmonary tuberculosis is generally regarded as a simple matter in which there is slight chance of error, there is a definite percentage of errors made in diagnosing the disease in this stage. They found that 72 out of a series of 1200 consecutive cases admitted to the Jefferson Hospital as advanced pulmonary tuberculosis were incorrectly diagnosed.

In the quest for a reliable method for this diagnosis, numerous investigators have repeatedly attempted to apply to the diagnosis of tuberculosis methods on which the diagnosis of other infectious diseases are based, such as the agglutinin, precipitin, meiostagmin and epiphanin reactions. Their attempts were crowned with partial success only, as the tests were found to be of little diagnostic value.

The discovery of the tubercle bacillus by Koch in 1883 placed the diagnosis of tuberculosis on a substantial foundation, but the bacillus cannot always be demonstrated early in the discharges, frequently never appearing, and even if present giving little clue to the degree of activity or inactivity of the disease. Thus far biologic methods of diagnosis have been of little practical value with one exception—complement fixation. Though not fulfilling the early expectation, this method of diagnosis has been gradually improved so that there was promise of its becoming as valuable a diagnostic test as the Wassermann reaction in syphilis.

The remarkable usefulness of the Wassermann reaction especially stimulated the efforts of numerous investigators along the lines of application of the Bordet and Gengou reaction to the diagnosis of tuberculosis. The earlier efforts along this line were not very encouraging. This could be due to several causes. It is possible that (due to the walled-off nature of the lesions and the slow process of the disease in certain cases) there may be no immune bodies or only a very few of these present in the circulation. Moreover, the concentration of circulating antibodies is subject to constant and quite marked fluctuation in the same patient. Besides, the antibodies in tuberculosis may not be, to any great extent, of the nature of amboceptor. Again, tuberculosis amboceptor, as suggested by Davidowitch, may be more thermolabile than most others, and since complement deviation was usually performed with inactivated serum, the amboceptor may have been largely destroyed in heating; thus the amount remaining in the serum may not have been large enough to be detected even by the delicate method of complement fixation.

This complement fixation reaction for tuberculosis has occupied, for nearly twenty years, the attention of many investigators who have studied it mainly from the standpoint of its possible value as a diagnostic and prognostic aid in clinical medicine.

Results obtained by different workers show considerable variation. When, however, it is remembered that the value of the test is entirely dependent on the specific action of the antigen and that the antigens used by many investigators have been prepared by widely divergent methods, it is to be expected that the results would be somewhat dissimilar. At best, we are but feeling our way toward a common understanding of the relation which our laboratory findings bear to the disease itself, and the test will eventually be established as of practical value only by a thorough comparison of all careful investigations rather than by the consideration of the work of one person.

In the diagnosis of tuberculosis it is not to be expected nor desired that the complement fixation test should replace the ordinary examination of sputum for tubercle bacilli, but if it is to be of any practical value to the clinician, a positive reaction must be specific. With these facts in mind, the writer has studied this complement fixation reaction to determine its value as a routine diagnostic test for tuberculosis.

In this work, the following questions have been taken up. First, what is the best general technic to employ for this test? Is special complement a necessity? What is the optimum fixation time and temperature, and is it necessary to keep the patient's serum for any period of time before testing? Secondly, what antigen will give the highest possible percentage of specific positive reactions and none or only an occasional non-specific reaction? Thirdly, is there any advantage in using the fresh non-inactivated serum with its native complement and amboceptor, or in removing the natural amboceptor from the inactivated serum? Fourthly, will the tubercle bacillus antigens used in this study give any non-specific cross fixation with syphilitic sera? Fifthly, is there any relationship of the complement fixation test for tuberculosis to the age, temperature, pulse or respiration of the patient? Sixthly, what is the best method for classifying tuberculosis patients from the combined view-point of the serologist and the clinician? Seventhly, what type of tuberculosis case gives the highest percentage of positive reactions? And lastly, just what value is this reaction going to be to the clinician as an aid to diagnosis and prognosis?

In this study the writer has made 6128 complement fixation tests on 1207 sera taken from 1000 patients. Of these sera 633 were from 484 patients in tuberculosis hospitals. The remaining 574 sera were from 516 patients in general hospitals suffering from various other diseases. There were made 1167 comparative tests with sera from 60 patients with 20 different tubercle bacillus antigens. Tests were made with 60 sera by the Hecht-Gradwohl technic and by the technic of absorbing out their natural antisheep amboceptor.

2. HISTORICAL REVIEW

In 1901 Bordet and Gengou described a method for detecting the presence of specific antibodies in the serum by means of complement fixation. Five years later the principle of this method was successfully applied by Wassermann, to the serum diagnosis of syphilis.

Widal and Le Sourd (2) appear to be the first who used the complement fixation reaction in attempting to arrive at a more certain method of diagnosing tuberculosis. Bordet and Gengou (3) in 1903 demonstrated the presence of antibody capable of uniting with tubercle bacilli and fixing complement in the sera of tuberculous animals. Wassermann and Bruck (4) in 1906 also demonstrated the presence of an antibody to tuberculin in patients treated with tuberculin.

Caulfield (5) and Beattie (6) in 1911, using bacilli emulsion as antigen, obtained 33 per cent positive reactions in primary tuberculosis, 70 per cent in moderately advanced cases and 62 per cent in far advanced tuberculosis.

Deilman (7) in 1911, using carbolized emulsion of tubercle and other acid-fast organisms as antigen, obtained fixation with tuberculous serum, the other acid-fast organisms giving about the same results as the tubercle bacilli.

Laird (8) in 1912, using a watery emulsion of tubercle bacilli, obtained fixation in only 4 out of 34 cases.

Mollers (9) in 1912, concluded that the fixation reaction permits no diagnostic or prognostic conclusions.

Hemmer (10) in 1912, using old tuberculin as antigen, obtained 100 per cent positive reactions on 48 tests of tuberculous cows and only 4 per cent non-specific reaction with non-tuberculous cows. He also reports (6) 97 per cent positive results with human tuberculous sera.

Zweig (11) in 1912, using a bacillen emulsion as antigen reported that his fixations were proportional to the severity of the disease.

Calmette and Massol (12) in 1912, using watery and dialyzable extract antigens reported fixation in 92.5 per cent of their cases.

Much (13) in 1912, using various acid-fast bacteria as antigens, with sera from tuberculous and healthy persons, obtained fixation in 77 per cent of the healthy cases, in other words, a large number of non-specific fixations.

Letulle (14) in 1912, using Calmette's antigens, obtained 89 per cent of fixation in tuberculous cases.

Fraser (15) in 1913, using various antigens, found that 96.6 per cent of sera from normal individuals gave no fixation with antigens made from living bacilli, and that with this antigen 42.3 per cent of sera from tuberculous individuals gave positive results. She states that the most reliable antigen is prepared from living human bacilli, and thinks that the complement-fixation test made with living bacillary antigen is of more value in the diagnosis of tuberculosis than any other reaction thus far discovered.

Dungeon, Meek and Weir (16) in 1913, report 85 per cent positive reactions with tuberculous patients not given specific treatment. Tuberculous patients treated with tuberculin 100 per cent positive, arrested cases 75 per cent positive. They conclude that killed tubercle bacillus emulsion makes the best antigen.

Bank and Anderson (17) in 1913, using emulsions of killed tubercle bacilli as antigen, obtained a marked per cent of strong positive reactions.

Ammand (18) in 1913, using crude tuberculin, obtained only 4 per cent definitely positive reactions while with a peptone soluble antigen they obtained 92 per cent definite reactions.

Wyschellesky (19) in 1913, using an emulsion of tubercle bacilli and a solution of the bacilli in 2 per cent lactic acid, obtained 18.1 per cent positive results with tuberculous cattle and also obtained 9.7 per cent positives with healthy cattle.

Kinghorn and Twitchell (20) in 1913, using a bacillus emulsion as antigen, reported 37.5 per cent positive in the incipient stage, and 93 per cent in advanced tuberculosis, with no fixations in normal cases. However, they tested only 33 cases altogether.

Rothe and Bierbaum (21) in 1913, reported that they did not obtain strong fixation with tuberculous cattle before treatment except in a few cases.

Harris and Lanford (22) in 1913, attempting to differentiate acid fast bacilli by means of the complement fixation test concluded that regardless of the various methods used to produce these sensibilizators no clean-cut specificity for complement fixation was found for the acid fast bacilli which they made use of in their experiments. They produced their anti-substances by injecting rabbits with whole bacilli or extract of the bacilli.

Momose (23) in 1913, using bodies of tubercle bacilli, after extraction of the fats, as antigen, obtained 100 per cent positive reactions with all tuberculosis patients and in nearly all exposed to the disease as well as 50 per cent in healthy persons.

Besredka (24) in 1914, using his special antigen, reported that all first stage tuberculosis patients react positive and nearly all second stage cases, but that in the third stage often partial or negative reactions were obtained.

Wwednesky (25) in 1914, using various tuberculins as antigen, reported 82.8 per cent positive and 16.2 per cent doubtful reactions in tuberculous cases. Ten non-tuberculous cases were negative.

Debains and Jupille (26) in 1914 using Besredka's antigen reported that the reaction is very sensitive in all forms of tuberculosis except miliary and meningeal and absent in healthy non-tuberculous people.

Kuss, Leredde and Rubinstein (27) in 1914, using Besredka's antigen, reported 89 per cent positive reactions in well-developed cases of pulmonary tuberculosis, about 66 per cent in mild cases and negative in all normals except those giving positive Wassermann reactions.

Inman (28) in 1914, using Besredka's antigen, reported 95 per cent positive reactions in 100 cases of pulmonary tuberculosis and 24 per cent positive in non-tuberculous patients.

Bierbaum and Berdel (29) in 1914, using bovine old tuberculin as antigen with the serum of 120 slaughtered cattle stated that the autopsy findings and serological findings agreed in only 65 per cent of the cases.

McIntosh and Fildes (30) in 1914, using fresh living tubercle bacilli as antigen, reported 76.7 per cent of positive reactions in pulmonary tuberculosis, 80.7 per cent in surgical tuberculosis (not glands) and no positives in the controls.

Radcliffe (31) in 1914, using the same antigen as McIntosh and Fildes, reported about 85 per cent positives with tuberculosis cases.

Dudgeon, Meek and Weir (32) in 1914, using an alcoholic extract of tubercle bacilli, reported 89 per cent positive reactions with tuberculosis cases when repeated examinations were made.

Meek (33) in 1914, stated that the greatest amount of antibody was found in severe cases with extensive lesions. Cases similar clinically may give different reactions.

Bronfenbrenner in 1914, in several communications (34, 35, 36, 37) on the use of Besredka's antigen, reported 93.84 per cent positive reactions in active tuberculosis. He stated that Besredka's antigen is specific, and syphilitic and tuberculous antibodies occurring in the same patient's serum are distinct and separable. Forty-three per cent of syphilitics gave positive reactions with Besredka's antigen, indicating an undue prevalence of tuberculosis in this class of patients.

Craig (38) in 1915 concluded that complement binding bodies are present in the blood serum of both active and inactive tuberculous infections. His polyvalent antigens prepared from several strains of tubercle bacilli have been found by him to give excellent results in the complement fixation test for tuberculosis. He obtained positive results in 96.2 per cent of active tuberculosis and 66.1 per cent in clinically inactive cases. The tests were negative with all his normal individuals, and with patients suffering from other diseases, with the exception of two patients infected with syphilis in whom symptoms of a coincident tubercular infection were also present.

Stimson (39) in 1915, using a variety of antigens, reported a small number of cases with but fair results. Corper (40) in 1916, using an autolysate as antigen and also a bacillary emulsion antigen, concluded that the complement fixation test for tuberculosis is not absolute, being positive in only about 30 per cent of all clinically definite cases of tuberculosis, both active and inactive.

Miller and Zinsser in 1916, in a communication to the New York Pathological Society, reported 100 per cent positive results in active cases and 100 per cent negative results in non-tubercular controls. In a subsequent communication (41) they reported 98.5 per cent positive reactions in active cases of tuberculosis and 32 positive fixations with 140 doubtful cases (i.e., patients suffering from diseases clinically diagnosed as other than tuberculosis). In a still later communication (42) on the clinical value of complement fixation in tuberculosis, Miller reported 96.8 per cent of positive results in active cases, 100 per cent negative results in non-tubercular and normal patients, and about 90 per cent negative results in inactive cases.

Woods, Bushnell and Maddux (43) in 1917 employing partial antigens (i.e., alcoholic extract antigens prepared by disintegrating the bacilli with 1 per cent lactic acid, filtering and extracting with alcohol) obtained positive results with 90 per cent of sera from cases classed as incipient, 87 per cent from active cases and 92 per cent from advanced tuberculosis cases.

McCaskey (44) in 1917 stated that specific complement-binding bodies were present in the blood of tuberculous patients, but not constantly so, even in clinically active cases; on the other hand, they may be present in cases having no clinical manifestations. These bodies, when present, may be demonstrated by the usual complement-fixation technic, and proves the existence of a focus which is pathologcally active. A negative fixation test does not absolutely exclude clinically active tuberculosis. The results of the tests in which tuberculins or bacillary suspensions were used as antigens are probably as dependable as the subcutaneous tuberculin test, and removes that element of danger to the patient which may be caused by the latter test. When the blood of the patient gives positive reactions with the tuberculosis complement-fixation test, and the Wassermann test, both tuberculous and luetic foci are present.

Brown and Petroff (45) in 1918, in a study correlating clinical and laboratory experience, found the test of greater value to them as a control of the therapeutic regimen than as a diagnostic measure. It parallels the subcutaneous tuberculin reaction in that a negative reaction in the tuberculous individual is of more value than a positive one in determining which patients need treatment. They have found the reaction of value, too, in pre-determining which patient will be benefited and which harmed by exercise and activity.

Lange (46) in 1918 reported fixation of some degree in 5 per cent of tuberculosis cases with 12.6 per cent in non-tuberculosis cases. He examined 864 serums with four different antigens, including Miller's and Petroff's potato broth culture antigen.

Stivelman (47) in 1918 reported on a series of 205 cases, 22 of which were non-tuberculous. Using Miller's antigen, he found that about 50 per cent of the tuberculous cases, active and inactive, gave positive fixations. He was unable to corroborate the favorable report of Miller.

Lewis (48) in 1919 has critically studied the reaction and has come to the conclusion that certain inherent defects will limit its usefulness. He suggested some modifications of the technic, however, such as increasing the time of fixation and increasing the quantities of complement and antigen in the effort to overcome the merely transient binding of complement that has possibly been interpreted by some workers heretofore as true deviation. It was his impression that it is unsafe to apply the reaction to the diagnosis of tuberculosis unless as a matter of confirmation of a clinical decision.

Pritchard and Roderick (49) in 1919, reported 69 per cent of positive reactions in active moderately advanced cases and 16 per cent of reactions in cases not proved to be tuberculosis. They thought that this test was a great aid in differential diagnosis.

Cooke (50) in 1919, concluded that in tuberculosis the serum contains complement binding substances that gave fixation in about 87 per cent of his cases.

Stoll and Neuman (51) in 1919, concluded that, from their experience, it would seem that with suspicious symptoms and suggestive, yet with inclusive signs, a negative fixation test, using the method described in this study, increases to a considerable degree the probability of the non-tuberculous nature of a given case. With the same symptoms and signs a persistently positive reaction probably signifies an active tuberculosis. A positive reaction occurring with neither symptoms nor signs does not justify a diagnosis of active tuberculosis, though it is quite probable that there has been an active process recently. In such a case, roentgenoscopy should be employed and the patient observed for several months.

To show how the opinion of laboratory workers in regard to this complement fixation test is divided even today, one needs only mention Mourseend's (52) conclusions as written in 1920. He stated that the complement fixation test for tuberculosis as described in his article is of no value as a diagnostic or prognostic aid, that this complement fixation test with alcoholic extract of tubercle bacilli as antigen is not specific and that a large percentage of serums giving a positive Wassermann give fixation with tubercle bacillus antigens.

Mourseend used a methyl alcoholic extract of the tubercle bacilli containing all the alcohol soluble fats along with other alcohol soluble substances; therefore, it is not to be wondered at that he, like Corper, should get non-specific cross-fixations with syphilitic sera.

TABLE 1

Results of experiments by different workers

NAME	SUSPICIOUS		EARLY TUBERCULOSIS		MODERATELY ADVANCED		FAR ADVANCED		OTHER CONDITIONS		NORMALS	
	Cases	Per cent plus	Cases	Per cent plus	Cases	Per cent plus	Cases	Per cent plus	Cases	Per cent plus	Cases	Per cent plus
Caulfield				33.0		70.0		63.0				
Radcliffe				88.6		89.6		79.0			204	0
Miller	140	22.8	32	100.0	110	98.0	83	98.0	45	4.4	144	0
Inman	50	60.0			100	95.0			100	24.0		
Debains						90.3		81.3		17.3		3.2
Bronfenbrenner	50	72.0			65	93.8			375	8.0		
Craig			30	96.7	61	98.3	54	96.4	450	4.4	200	0.5
McCaskey	8	25.0			36	77.7			74	20.2	9	11.1
Petroff	20	65.0	64	81.2	123	91.0	2	100.0	14	7.0	14	0
Corper	69	43.6	28	35.7	61	70.5	63	61.0	31	19.3		
Moon	61	60.0	24	87.5	49	85.7	83	84.3	23	26.0	100	12.0
Calmette					134	92.5*						
Dudgeon					234	89.3*						
McIntosh						76.7*					87	3.5

* Various stages grouped.

Moon (53) has tabulated the experiments of the more important studies according to the general broad classification of tuberculosis patients. His summary is given in table 1.

Bronfenbrenner (54) in 1917 suggested that at least two reasons for the failure of advanced cases to give fixation can be offered tentatively; one is that the resistance of the patient having been exhausted, there is no new antibody formation; and the other, that the circulating antibody is taken up as formed by the combination with antigen which may greatly increase during the last stages of the disease.

Boez and Duhot (55) in 1919 said that if one considers the various stages of pulmonary tuberculosis, the curve of the antibodies, at first low, rises during the first and second period; that it is maintained or increased at the beginning of the third period; and at the ultimate phase, the antibodies can disappear in a rapid manner, in coincidence with the progress of the cachetic premonitories of death.

Depending on the antigen and the technic used, the percentage of tuberculosis cases giving positive fixations vary from about 95 per cent down to a low figure. While the antigens and technics giving the highest percentage of positive results are more valuable in confirming suspected cases or in detecting unsuspected ones, they tend to approach such tests as the von Pirquet in failing to give information as to the activity of the tubercular process and are therefore misleading as suggesting in any degree an active process.

It seems to be generally conceded that a large percentage of human individuals have had some tuberculous lesion during life. Many, however, become quiescent; all traces of the bacilli even disappearing. Those antigens and technics giving a lower percentage of positive findings are of more value in that they give positive evidence of the activity of the disease with practically no non-specific reactions.

The most significant feature of this summary is that, while there are differences in percentages due evidently to the different methods and reagents used, there is agreement that complement fixation under proper conditions gives positive results in the majority of cases of active tuberculosis. The laboratory technic employed in this test is the same in principle and in main details as in complement fixation applied to the diagnosis of syphilis. As in that test there have been many variations in the technic and in the reagents employed, and as would be expected, the results have also varied. The widest variation occurs in the preparation of the tubercle bacillus antigen. The reaction is one of biologic specificity depending on the presence in the patient's serum of free antibodies specific to tubercle bacilli. In this particular, the situation differs from that in syphilitic infection.

3. TECHNIC OF THE STANDARD COMPLEMENT FIXATION TEST FOR TUBERCULOSIS USED IN THIS STUDY

The technic employed was similar to that originally used by Wassermann with the following modifications.

At first the tests were carried out in both one-quarter and one-tenth the original Wassermann volume, but as the writer found no difference in the results, he has since continued to use the one-tenth Wassermann volume only.

Complement. The pooled blood serum from six to ten healthy guinea-pigs was used as complement; in addition, we used serum from separate guinea-pigs untested for its complement fixation value; serum from separate guinea-pigs after having been tested for complement fixation value and serum from six to eight guinea-pigs, all of which had been specially tested for complement fixation value. All these complements were titrated with a 2.5 per cent sheep cell suspension, sensitized with three units of antisheep amboceptor; the unit was recorded at the end of fifteen minutes. Exactly two units were used in the regular test. Many different balances of the hemolytic system have been tried but the most constant results have been with the two hemolytic units of selected complement combined with cells sensitized with three standard units of amboceptor.

Antigen. The Wilson antigen (56) which was used as the standard antigen throughout this study is a suspension in 0.9 per cent saline of dried bacilli, from which all constituents soluble in alcohol and ether have been removed. The bacilli were grown in glycerin-broth. The monovalent antigen cultures were grown for three months. The broth cultures were killed by heating them in the Arnold sterilizer for one hour. The cultures were then filtered through filter paper. The filtrate was discarded, and the residue was placed in absolute alcohol, in the proportion of one volume of residue to ten volumes of alcohol. This mixture was shaken thoroughly by hand, and was placed in the ice box for two weeks. It was then filtered through paper and the filtrate was discarded. The residue was washed in absolute alcohol and the sediment obtained by centrifugalization was washed in ether. After further centrifugation the ether was discarded and the centrifuge tube containing the residue was plugged and placed in the dark at room temperature over night. By this simple procedure the residue was dried within twenty-four hours. The dried powder was emulsified in a large mortar with 0.9 per cent saline in the proportion of 1 gram of powder in 200 cc. of saline. This gave a concentrated emulsion convenient for storing as a stock antigen. The emulsion was heated for one hour at 80°C. The antigen was then ready for use, and it was standardized to be used in such a dilution that 0.1 cc. contained two standard fixation units and one-fourth, or less, of the anticomplementary dose. The unit was determined by titrating varying amounts of the antigen with 0.01 cc. of a known positive tuberculosis serum, and two hemolytic units of a complement known to be potent for tuberculosis fixation. The standard dilution of the antigens employed is usually 1:50. This makes a final dilution of dried bacilli 1:10,000. These antigens are not anticomplementary in the amount used in the test. They have given uniform and constant fixation reactions.

Sheep cells. A 5 per cent suspension of sheep cells, which had been washed five times in sterile saline was used, after having been sensitized with an equal volume of diluted amboceptor in the water bath for half an hour.

Amboceptor. Three units were used in the tests.

Fixation period. After the patient's serum, complement, antigen and saline were mixed, the mixtures were incubated in the water bath at 37.5°C. for one hour. The sensitized cells were then added and the reading was made in exactly fifteen minutes.

Results were reported as plus minus if any degree of fixation was observed; 1 plus if there was marked fixation in the first antigen tube; 2 plus if there was complete fixation in the first tube; 3 plus if there was complete fixation in the first tube and marked fixation in the second tube; and 4 plus if there was complete fixation in both tubes.

During 1913, Cyrus W. Field and the writer carried out a series of 730 Wassermann reactions in the Bellevue Hospital Laboratories, using the regular amount of serum prescribed by Wassermann and also twice, three times, four times and five times that amount. These amounts of patient's serum were tested in all of the 730 cases, our controls being carried out with double the amounts of serum used with the antigen. Discarding all those cases that were anticomplementary in the regular Wassermann amounts, and considering only those which ordinarily would be considered as not anticomplementary, we found that we had no anticomplementary and no non-specific reactions with double the usual amount of serum. Three times the usual amount of serum gave about 1 per cent of anticomplementary reactions, four times the usual amount of serum gave about 5 per cent of anticomplementary reactions and five times the usual amount of serum gave about 25 per cent anticomplementary reactions.

As these results were so favorable, and as several other investigators have made favorable reports on the use of larger quantities of patients's sera, the writer has made all his tests since January 1, 1918, with the regular Wassermann amount and with double that amount of patient's serum; that is, 0.04 cc. of serum in the first antigen tube with 0.08 cc. of serum in its control tube; 0.02 cc. of serum in the second antigen tube with 0.04 cc. of serum in the control tube and 0.01 cc. of serum in the third antigen tube.

However, it is perfectly possible to obtain approximately the same results by either of the two following methods:

First, using the regular amounts of patient's sera and antigen containing four antigenic units. Second, by using double the regular amounts of patient's sera and antigen containing two antigenic units.

The writer has found that if his antigen has a relatively small range between its fixation and its anticomplementary dose, it is best to double the amount of the patient's serum, and use only two antigenic units. If, however, one has a wide range with the antigen, the regular amount of patient's serum and four antigenic units can be used.

While at the Walter Reed Army Hospital, the writer experimented with the Noguchi system, using human red blood cells as the indicator instead of the sheep cells in order to do away with the troublesome feature of natural anti-sheep amboceptor. It was soon discarded, however, as the amboceptor was of a very low titer. This necessitated the use of a large amount of complement in the working system. Most of the results were negative with this system due probably to the use of this excess amount of complement, as the smallest excess of the regular amount of complement used very markedly reduces the percentage of positive findings. In fact, the complement factor appears to be the most important point to watch in making the complement fixation test for tuberculosis. Apparently the, complement fixation in this test is very much weaker than when one uses the lipoid antigens for the Wassermann test.

4. DETERMINATION OF THE OPTIMUM TIME AND TEMPERATURE OF FIXATION

During this study, tests have been made to determine the optimum time and temperature of the complement fixation period. In all, 135 comparisons at various fixation periods have been made as follows: One hour at 37°C. in the water bath; two hours at 37°C. in the water bath; two hours at 10°C. in the ice chest followed by two hours at 37°C. in the water bath; four hours at 10°C. in the ice chest; and over night at 10°C. in the ice chest.

The results of a few of these comparisons are given in table 2. Apparently the one hour water bath fixation period gives the most uniform results. The ice box fixation for longer periods of time gave very weak results. Two hours in the water bath gave marked increased fixation in a considerable number of cases, but there were more anticomplementary reactions than with the one hour fixation period. Two hours at 10°C. in the ice chest followed by two hours at 37°C. in the water bath gave about the same results as the two hour 37°C. fixation.

The above findings in regard to the weak fixation of tubercle bacillus antigens at ice box temperature are in variance with the findings of Ruediger (57) who investigated the optimum time and temperature for Wassermann fixations. He reported that fixation at 1°C. for twenty-four hours gave the strongest positive results when compared with fixations at various temperatures from 2° to 37°C., and from one hour to twenty-four hours. He concluded that it is advisable to warm the serum-complement antigen mixture before adding the sensitized blood corpuscles.

TABLE 2

Showing variations in the results of a few of the tests obtained by fixation at various times and temperatures

SERUM NUMBER	ONE HOUR* AT 37°C.				TWO HOURS AT 37°C.				TWO HOURS§ AT 10°C. AND 2 HOURS AT 37°C.				FOUR HOURS AT 10°C.			
	Control tubes		2 × regular† volume	Regular‡ volume.	Control tubes		2 × regular volume	Regular volume	Control tubes		2 × regular volume	Regular volume	Control tubes		2 × regular volume	Regular volume
	0.08 cc.	0.04 cc.			0.08 cc.	0.04 cc.			0.08 cc.	0.04 cc.			0.08 cc.	0.04 cc.		
201	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
205	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
206	–	–	4+	4+	–	–	4+	4+	–	–	4+	4+	–	–	4+	4+
208	–	–	3+	+	–	–	3+	+	–	–	3+	+	–	–	+	–
213	–	–	2+	+	–	–	4+	2+	–	–	4+	2+	–	–	+	–
214	–	–	4+	4+	–	–	4+	4+	–	–	4+	4+	–	–	3+	2+
216	–	–	4+	2+	–	–	4+	3+	–	–	4+	3+	–	–	2+	±
219	–	–	4+	4+	+	±	4+	4+	+	±	4+	4+	–	–	4+	4+
220	–	–	3+	+	–	–	4+	2+	–	–	3+	2+	–	–	+	–
221	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
231	–	–	4+	3+	–	–	4+	3+	–	–	4+	3+	–	–	3+	1+
233	–	–	–	–	–	–	±	–	–	–	+	–	–	–	–	–
235	–	–	–	–	–	–	±	±	–	–	+	–	–	–	–	–
246	+	±	4+	2+	±	–	4+	4+	+	–	4+	2+	+	±	4+	4+
247	–	–	2+	+	–	–	4+	4+	–	–	3+	2+	–	–	–	–
407	–	–	2+	+	±	–	2+	+	+	–	4+	+	–	–	+	+
409	–	–	3+	±	±	–	4+	2+	–	–	3+	+	–	–	2+	±

* Refers to the fixation time in the water bath at 37°C.

† Refers to the use of double the regular Wassermann amount of patient's serum.

‡ Refers to the use of the regular Wassermann amount of patient's serum.

§ Refers to the fixation time in the ice box at 10°C.

5. THE STANDARDIZATION OF COMPLEMENT

M. A. Wilson (56) has observed that the serum of a number of guinea-pigs is unsuitable for use in the complement fixation test for tuberculosis because the complement of these guinea-pig sera is not fixed, under the usual conditions of the test with the sera of tuberculous individuals.

A similar irregularity in the guinea-pigs' sera, when employed in the Wassermann test, was reported by Noguchi and Bronfenbrenner (58) in their article on the "Variation of the complement activity and fixability of guinea-pigs' sera in Wassermann work."

They state that positive patient's sera will often fix complement from some guinea-pigs and will not fix complement from other guinea-pigs, but that there is no definite relationship between the complementary activity and the fixability of a given specimen of guinea-pig's serum. However, the irregularity observed by the latter authors was relatively infrequent as compared with that reported by M. A. Wilson.

With the purpose of further studying this phenomenon, the writer carried out a number of tests with sera from actively tuberculous patients; at first, in two series, one with pooled guinea-pigs' sera that had not been tested as to fixability, the other with pooled guinea-pigs' sera, each of which had been separately so tested and found satisfactory. The pooled serum was derived, in each case, from six to eight guinea-pigs. These parallel tests gave practically the same results. In a few instances, the writer obtained a 2 plus reaction with the tested complement instead of a 1 plus reaction with the untested complement, or a 3 plus instead of a 2 plus reaction; but this difference was not regularly encountered and in a few instances, in fact, better results were obtained with the pooled untested complement than with the specially tested complement.

This experiment being inconclusive, the writer then carried out a series of tests with sera from seven frank tubercular cases (six of which he had already examined) testing each guinea-pigs complement separately against each positive serum. The results of these tests are shown in table 3.

Serum 1, tested with complement from guinea-pig 1, gave negative results in the antigen tubes containing 0.02 cc. and 0.04 cc. of patient's serum on the first and fourth days after taking the specimen from the patient. On the sixth day and again on the seventh day, complement from guinea-pig 2 gave a 3 plus reaction with 0.02 cc. of patient's serum and a 4 plus reaction with double that amount. Tests made on the ninth and eleventh days with complement from guinea-pig 3 gave negative results with all amounts of patient's serum. On the fourteenth day, the pooled complement, made from sera of six tested guinea-pigs, gave results which were practically the same as those obtained when complement from guinea-pig 2 was used; that is, a 2 plus reaction with 0.01 cc. and 0.02 cc. of patient's serum and a 4 plus reaction with 0.02 cc. and 0.04 cc. of patient's serum. On the sixteenth day, complement from an additional guinea-pig which may be designated as no. 4 gave a plus-minus and a 1 plus reaction.

TABLE 3

Showing variations in flexibility of the complement of different guinea-pigs' sera in the complement-fixation test in tuberculosis

PATIENT'S SERUM NUMBER	COMPLEMENT NO. 1 FIRST TEST		COMPLEMENT‡ NO. 1 LATER TEST		COMPLEMENT NO. 2 FIRST TEST		COMPLEMENT‡ NO. 2 LATER TEST		COMPLEMENT NO. 3 FIRST TEST		COMPLEMENT‡ NO. 3 LATER TEST		POOLED TESTED COMPLEMENT FIRST TEST	
1	–*	–†	±	–	4+	2+	4+	3+	–	–	–	–	4+	2+
2	–	–	–	–	4+	3+	4+	3+	1+	–	–	–	4+	2+
3	–	–	–	–	4+	2+	3+	2+	–	–	–	–	1+	±
4	–	–	–	–	4+	4+	4+	4+	1+	–			4+	3+
5	2+	–	3+	1+	4+	4+	4+	4+	4+	–	4+	–	4+	4+
6	–	–	–	–	4+	1+	3+	1+	–	–	–	–	±	–
7	–	–	2+	–	4+	4+	4+	4+	–	–	–	–	3+	1+

* The results in the first column were obtained with 0.04 cc. of patient's serum.

† The results in the second column were obtained with 0.02 cc. of patient's serum.

‡ Complement was preserved in the interim with an equal amount of 18 per cent salt solution and kept in contact with ice.

Patients' sera 2, 3, 4, 6 and 7 gave almost identical results.

Serum 5 gave a 1 plus and a negative result with complement 1, a 4 plus reaction with complement 2, negative results with complement 3, a 4 plus reaction with the pooled complement, and a 1 plus reaction with complement 4, when the regular amount of patient's serum was used.

These observations seem to confirm the statements of M. A. Wilson, Noguchi and Bronfenbrenner on the variation in complement activity and fixability of guinea-pigs sera, but the use of pooled complement from six to eight guinea-pigs as a rule apparently overcomes this variability of separate guinea-pig sera.

In studying the difference in the first and seventh day tests and also in studying the loss of natural anti-sheep hemolysins in the patients' serum due to ageing, the writer used a salted complement. A sufficient quantity of this salted complement was made up to last for all these comparative tests so that no differences in the complement would complicate the results. After studying the various methods of preserving complement including Ronchese's (59)—sodium fluoride method, Thompson's (60)—sodium chloride method, Rhamy's (61)—sodium acetate method, and McNeill's (62)—freezing method, the writer concluded from many experiments that simply adding 8.5 per cent of dry powdered salt to the pure guinea-pig serum, and keeping the tube (not necessarily sterile) in a thermos bottle packed with plain cracked ice, preserved the complement for at least one month without the slightest loss of complementary activity.

6. DETERMINATION OF THE THERMOLABILITY AND THE THERMOSTABILITY OF ANTI-SHEEP CELL HEMOLYSINS IN HUMAN SERA

To determine whether or not the natural anti-sheep hemolysin in human sera is thermolabile or thermostabile, the writer has made hemolysin titrations on 187 different sera. These have all been titrated after being heated in the water bath at 56°C. for various periods of time. All received the initial thirty minutes inactivation and were then reheated for fifteen, thirty, forty-five, sixty or one hundred and twenty minute periods in different lots. On 97 of these sera, these heat tests were repeated after keeping the sera for seven days under sterile conditions. These kept sera were inactivated the first day for the regular one-half hour period at 56°C., and were then stored in the ice box. On the seventh day the titrations were repeated after reheating the sera in separate tubes for various periods of time as in the previous titrations. The volumes of serum used in most of these titrations were 0.01 cc., 0.02 cc., 0.04 cc., 0.06 cc., 0.08 cc., and 0.1 cc. At first, as high as 0.16 cc. was used but the volumes above 0.1 cc. were so frequently anticomplementary that later only from 0.01 to 0.1 cc. were used in the titrations. These volumes gave a wide range and proved very satisfactory. The complement used in these titrations was all tested for natural anti-sheep amboceptor and any complement showing the slightest trace of hemolysis was discarded. All titrations were set up with two units of complement. The complement volume necessarily varied as one day the serum volume of the one unit of complement would be 0.04 cc. and possibly the next week it would be 0.06 cc. Also the 0.06 cc. might have been just exactly one unit while the 0.04 cc. was possibly a strong one unit (1.2 units) as the complement titrations were regularly made with 0.01, 0.02, 0.03, 0.04, 0.05 and 0.06 cc. volumes. The unit therefore would be somewhere between 0.03 and 0.04 cc.

One-tenth of a cubic centimeter of a 5 per cent suspension of washed sheep cells was added to each tube. Cells from a different sheep were used each week. Consequently, some were more resistant to hemolysin than others. These variations in the blood cells and the complement probably account for some of the apparent discrepancies noted in the results of the first and the seventh day titrations.

In going over the results of these titrations one at once notes the marked loss of hemolysin in the heated sera. This loss seems to be progressive; i.e., the longer the sera are heated the greater is the loss of hemolysin. In table 4, eight sera show no hemolysis in the tests after being reheated one hour at 56°C.; four show a lesser degree of hemolysis and three contained such an excess of hemolysin that a loss was not indicated by the tests.

In tables 5, 6, and 7 this loss of hemolysin is also noted to a more or less degree. Even fifteen minutes extra heating caused a slight loss of hemolysin. In table 6 the sera kept for seven days seemed to show a greater loss of hemolysin due to heating forty-five minutes than the fresh sera heated the same length of time. Table 7 does not show any great loss of hemolysin as the extra period of heating was only fifteen minutes. However, heating at 56°C. even for two hours does not destroy all the hemolysin present in human sera. In table 4 we see that sera nos. 59N, 70N, 71N, 77N, and 90N still show hemolysis in the tests to a greater or less degree after the two hours heating. This seems to point to the presence of a thermostable hemolysin which may cause complete hemolysis if a sufficient quantity of complement is present.

TABLE 4

Showing the loss of natural antisheep hemolysin due to heating for long periods at 56°C. in the water bath

SERUM NUMBER	TEN MINUTES AT 56°C.						ONE HOUR AT 56°C.						TWO HOURS AT 56°C.					
	0.01 cc.	0.02 cc.	0.04 cc.	0.06 cc.	0.08 cc.	0.10 cc.	0.01 cc.	0.02 cc.	0.04 cc.	0.06 cc.	0.08 cc.	0.10 cc.	0.01 cc.	0.02 cc.	0.04 cc.	0.06 cc.	0.08 cc.	0.10 cc.
4086	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg						
4088	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg						
4089	sf	wf	wf	tf		neg	sf	wf	wf	wf	tf	neg						
4092	wf	tf	tf	tf	wf	sf	sf	wf	wf	wf	sf	cf						
5004	wf	tf	tf	tf	tf		cf	cf	cf	cf	cf							
4094	cf	wf	neg	neg	neg	neg	cf	wf	tf	neg	neg	neg						
4096	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg						
4097	neg	neg	neg	neg	neg	neg	tf	neg	neg	neg	neg	neg						
5035	tf	neg	neg	tf	neg	neg	cf	cf	cf	cf	cf		cf	cf	cf	cf	cf	
5051	cf	neg	neg	neg	neg		cf	cf	cf	cf	cf		cf	cf	cf	cf	cf	
5062	tf	tf	neg	neg	neg		cf	cf	cf	cf	cf		cf	cf	cf	cf	cf	
5080	cf	wf	wf	tf	wf		cf	cf	cf	cf	cf		cf	cf	cf	cf	cf	
5089	cf	cf	cf	cf	cf		cf	cf	cf	cf	cf		cf	cf	cf	cf	cf	
5093	tf	neg	neg	neg	neg		cf	cf	cf	cf	cf		cf	cf	cf	cf	cf	
5096	wf	tf	neg	neg	neg		cf	cf	cf	cf	cf		cf	cf	cf	cf	cf	
5099	tf	neg	neg	neg	tf		cf	cf	cf	cf	cf		cf	cf	cf	cf	cf	
5101	cf	cf	cf	cf	cf		cf	cf	cf	cf	cf		cf	cf	cf	cf	cf	
59N	neg	neg	neg	neg	neg	neg							wf	tf	neg	neg	tf	wf
62N	cf	cf	cf	sf	cf	cf							cf	cf	cf	cf	cf	cf
70N	wf	tf	neg	tf	wf	wf							sf	wf	wf	wf	sf	sf
71N	wf	wf	neg	neg	neg	neg							wf	wf	wf	tf	wf	wf
77N	sf	wf	neg	neg	neg	neg							wf	tf	neg	neg	neg	sf
90N	wf	wf	neg	neg	neg	neg							cf	tf	neg	neg	tf	tf

NOTE: Negative refers to neg; tf, trace of fixation; sf, strong fixation; cf, complete fixation.

TABLE 5

Showing the loss of natural antisheep amboceptor in human sera due to heating for moderate periods at 56°C.

NUMBER	NOT REINACTIVATED*						REINACTIVATED FIFTEEN MINUTES AT 56°C.						REINACTIVATED FORTY-FIVE MINUTES AT 56°C.					
	0.01 cc.	0.02 cc.	0.04 cc.	0.06 cc.	0.08 cc.	0.10 cc.	0.01 cc.	0.02 cc.	0.04 cc.	0.06 cc.	0.08 cc.	0.10 cc.	0.01 cc.	0.02 cc.	0.04 cc.	0.06 cc.	0.08 cc.	0.10 cc.
4603†	neg‡	neg	neg	neg	neg	neg	wf	tf	neg	neg	tf	neg	sf	wf	wf	tf	tf	wf
4605	tf	neg	neg	neg	neg	neg	wf	neg	neg	wf	neg	tf	sf	tf	neg	tf	neg	tf
4613	neg	neg	neg	tf	neg	tf	tf	tf	neg	tf	tf	tf	sf	wf	tf	tf	tf	wf
4614	sf	tf	tf	tf	tf	tf	sf	wf	tf	tf	tf	tf	wf	tf	neg	neg	neg	tf
4620	neg	neg	neg	neg	neg	neg	wf	wf	wf	wf	wf	sf	cf	wf	wf	wf	wf	sf
4622	tf	neg	neg	tf	tf	tf	tf	neg	neg	neg	neg	neg	cf	tf	tf	neg	neg	neg
4625	neg	neg	neg	neg	neg	0	neg	neg	neg	neg	neg	neg	tf	neg	neg	neg	neg	neg
4628	neg	neg	neg	neg	neg	0	neg	neg	neg	tf	tf	tf	cf	neg	tf	tf	tf	wf
4629	tf	neg	neg	neg	neg	0	tf	neg	tf	neg	tf	tf	sf	wf	tf	neg	tf	tf
4649	tf	0	neg	neg	neg	0	wf	tf	wf	tf	tf	wf	sf	wf	tf	0	sf	0
4650	neg	neg	neg	neg	neg	0	tf	neg	neg	neg	neg	neg	cf	wf	neg	neg	0	tf
4651	tf	neg	neg	neg	neg	0	cf	cf	wf	neg	tf	tf	cf	cf	cf	cf	cf	cf
4653	neg	neg	neg	0	0	0	cf	cf	wf	neg	0	0						
4654	0	0	0	0	0	0	sf	sf	neg	wf	neg	tf	sf	wf	neg	neg	neg	
4657				tf	tf	sf				tf	tf	wf				tf	cf	cf
4658				sf	tf	sf				wf	wf	sf				sf	cf	cf

* All these sera had been inactivated the usual one half hour at 56°C. the day previously.

† These tests were made on August 1, 1919.

‡ Neg refers to negative; tf, trace of fixation; wf, weak fixation; sf, strong fixation, and cf, complete fixation.

TABLE 6

Showing the loss of natural antisheep hemolysin in human sera due to heating at 56°C.

NUMBER	REINACTIVATED TEN MINUTES						REINACTIVATED TWENTY-FIVE MINUTES						REINACTIVATED FIFTY-FIVE MINUTES					
	0.01 cc.	0.02 cc.	0.04 cc.	0.06 cc.	0.08 cc.	0.10 cc.	0.01 cc.	0.02 cc.	0.04 cc.	0.06 cc.	0.08 cc.	0.10 cc.	0.01 cc.	0.02 cc.	0.04 cc.	0.06 cc.	0.08 cc.	0.10 cc.
4603	neg	neg	neg	neg	neg	neg	wf	tf	tf	neg	tf	tf	cf	wf	wf	cf	cf	cf
4605	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	tf	tf	tf	neg	tf	sf
4610	wf	tf	neg	neg	neg	neg	sf	wf	wf	wf	sf	sf	cf	cf	cf	cf	cf	cf
4611	tf	neg	neg	neg	neg	neg	sf	wf	wf	wf	sf	cf	cf	cf	cf	cf	cf	cf
4613	neg	neg	neg	neg	neg	neg	tf	neg	neg	neg	neg	tf	sf	sf	sf	cf	cf	cf
4614	wf	tf	neg	neg	neg	neg	wf	tf	neg	neg	neg	tf	cf	sf	wf	wf	wf	wf
4620	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	tf	neg	neg	neg	neg	neg
4622	neg	neg	neg	neg	neg	neg	wf	tf	neg	neg	tf	tf	cf	wf	wf	wf	wf	sf
4625	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	tf	neg	neg	tf	tf	tf
4628	neg	neg	neg	neg	neg	neg	tf	neg	neg	neg	neg	neg	tf	tf	tf	tf	wf	wf
4629	neg	neg	neg	neg	neg	neg	wf	tf	tf	tf	tf	tf	cf	cf	cf	cf	cf	cf
4634	neg	neg	neg	neg	neg	neg	wf	tf	tf	tf	tf	tf	cf	cf	cf	cf	cf	cf
4646	neg	neg	neg	neg	neg	neg	wf	neg	neg	neg	neg	neg	wf	tf	tf	wf	wf	sf
4648	tf	tf	neg	neg	neg	neg	tf	tf	tf	tf			wf	tf	0	0	0	
4650	neg	neg	neg	neg			neg	neg	neg	neg			tf	neg	neg	neg		
4651	tf	neg	neg	neg	neg	neg	wf	tf	tf	tf	tf	tf	cf	cf	cf	cf	cf	cf
4654	neg	neg	neg	neg			neg	neg	neg	neg			tf	neg	neg	tf	0	0
4657	tf	neg	neg	neg	tf		tf	tf	tf	wf	sf	0	wf	tf	sf	cf		
4658	tf	tf	neg	neg	tf	tf	sf	tf	sf	cf	cf	cf	cf	cf	cf	cf	cf	cf

* These sera had been inactivated for thirty minutes on July 31, 1919.

† These tests were made on August 7, using the sera which had been tested on August 1, 1919 (part of each specimen of sera was set aside for these later tests).

NOTE: By comparing the results of these tests with those tabulated on table V, one will see that only a moderate loss of hemolysin took place due to the ageing of the sera. This was only noticeable in the fifty-five minute inactivation tests.

TABLE 7

Showing the loss of natural antisheep hemolysins in human sera due to short periods of heating and to ageing the sera

SERUM NUMBER	FIRST DAY* NOT REINACTIVATED						FIRST DAY FIFTEEN MINUTES REINACTIVATED					
	0.01 cc.	0.02 cc.	0.04 cc.	0.06 cc.	0.08 cc.	0.10 cc.	0.01 cc.	0.02 cc.	0.04 cc.	0.06 cc.	0.08 cc.	0.10 cc.
31	cf	cf	cf	cf	cf	cf	cf	cf	cf	cf	cf	cf
33	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
34	wf	neg	neg	neg	neg	neg	sf	tf	neg	neg	neg	neg
35	neg	neg	neg	neg			neg	neg	neg	neg		
36	sf	sf	sf	sf			sf	wf	sf	sf		
37	neg	tf	neg	neg			wf	neg	neg	neg		
38	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
39	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
40	sf	tf	neg	neg	neg	neg	wf	tf	tf	neg	neg	neg
41	wf	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
42	neg	neg	neg	neg	neg	neg	tf	neg	neg	neg	neg	neg
43	tf	neg	neg	neg	neg	neg	wf	neg	neg	neg	neg	neg
44	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
45	neg	neg	neg	neg	neg	neg	tf	neg	neg	neg	neg	neg
51	sf	neg	neg	neg	neg	neg	tf	neg	neg	neg	tf	wf
52	cf	cf	cf	cf			cf	cf	cf	cf		
53	tf	neg	neg	neg	neg	neg	tf	tf	neg	neg	neg	neg
54	cf	cf	cf	cf	cf	cf	cf	cf	cf	cf	cf	cf
55	cf	cf	cf	cf	cf	cf	cf	cf	cf	cf	cf	cf
60	neg	neg	neg	neg	neg	neg	tf	neg	neg	neg	neg	neg
	SEVENTH DAY NOT REINACTIVATED						SEVENTH DAY FIFTEEN MINUTES REINACTIVATED					
31	cf	cf	cf	cf	cf	cf	cf	cf	cf	cf	cf	cf
33	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
34	wf	tf	neg	neg	neg	neg	wf	wf	neg	neg	neg	
35	neg	neg	neg	neg			neg	neg	neg	neg		
36	cf	cf	cf	cf	cf	cf	cf	cf	cf	cf	cf	cf
37	tf	neg	neg	neg	neg	neg	tf	tf	neg	neg	neg	neg
38	tf	neg	neg	neg	tf	tf	tf	neg	neg	neg	neg	neg
39	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
40	tf	neg	tf	tf	tf	wf	tf	neg	neg	neg	neg	tf
41	neg	neg	neg	neg	neg	tf	neg	neg	neg	neg	neg	tf
42	neg	neg	neg	neg			tf	neg	neg	neg		
43	tf	neg	neg	neg			tf	neg	neg	neg		
44	neg	neg	neg	tf			neg	neg	neg	neg	neg	neg
45	tf	neg	neg	neg	neg	neg	tf	neg	neg	neg	neg	neg
51	tf	neg	neg	tf			tf	neg	neg	neg	tf	
52	cf	cf	cf	cf	cf	cf	cf	cf	cf	cf	cf	cf
53	cf	neg	tf	wf	wf	wf	wf	tf	neg	tf	tf	tf
54	cf	cf	cf	cf	cf	cf	cf	cf	cf	cf	cf	cf
55	0	0	0	0	0	0	0	0	0	0	0	0
60	neg	neg	neg	neg	tf	tf	neg	neg	neg	neg	tf	tf

* The sera had been inactivated the usual thirty-minute period at 56°C. the day previously.

NOTE: Negative, neg; trace of fixation, tf; weak fixation, wf; strong fixation, sf; complete fixation, cf.

TABLE 8

On the loss of natural antisheep hemolysin due to ageing.

NUMBER	SERA TESTED, FIRST DAY*						SERA TESTED, SEVENTH DAY†						SERA TESTED, FOURTEENTH DAY‡					
	0.01 cc.	0.02 cc.	0.04 cc.	0.06 cc.	0.08 cc.	0.10 cc.	0.01 cc.	0.02 cc.	0.04 cc.	0.06 cc.	0.08 cc.	0.10 cc.	0.01 cc.	0.02 cc.	0.04 cc.	0.06 cc.	0.08 cc.	0.10 cc.
4512	cf	cf	cf	cf	cf	cf	cf	cf	cf	cf	cf	cf			neg		neg	
4513	cf	cf	cf	cf	cf	cf	cf	cf	cf	cf	cf	cf			cf		cf	
4516	tf	neg	neg	neg	neg	neg	tf	tf	neg	tf	wf	wf			neg		neg	
4519	cf	cf	cf	cf	cf	cf	cf	cf	cf	cf	cf	cf			cf		cf	
4520	sf	wf	tf	tf	tf	tf	sf	wf	wf	sf	sf	cf			tf		tf	
4521	cf	cf	cf	cf	cf	cf	cf	cf	cf	cf	cf	cf			cf		cf	
4523	wf	wf	wf	wf	wf	wf	sf	sf	sf	cf	cf	cf			wf		sf	
4524	wf	wf	wf	wf	cf	cf	cf	cf	cf	cf	cf	cf			wf		sf	
4525	cf	cf	cf	cf	cf	cf	cf	cf	cf	cf	cf	cf			cf		cf	
4528	tf	tf	tf	tf	tf	tf	sf	wf	sf	wf	sf	cf			neg		neg	
4531	cf	cf	cf	cf	cf	cf	cf	cf	cf	cf	cf	cf						
4536	cf	cf	cf	cf	cf	cf	cf	cf	cf	cf	cf	cf						
4539	cf	cf	cf	cf	cf	cf	cf	cf	cf	cf	cf	cf						
4542	cf	cf	cf	cf	cf	cf	cf	cf	cf	cf	cf	cf						
4547	wf	wf	wf	tf	tf	tf	wf	wf	wf	sf	cf	cf			wf		sf	
4548	sf	sf	wf	wf	sf	sf	cf	cf	cf	cf	cf	cf			cf		cf	
32B	cf	sf	wf	wf	wf	wf	cf	cf	sf	wf	wf	sf	neg	neg	neg	neg	neg	neg
34B	wf	tf	tf	neg	tf	neg	wf	wf	wf	tf	sf	sf	neg	neg	neg	neg	neg	neg

* Sera used on the first day were inctivated for the usual thirty minutes at 56°C.

† The sera were not reinactivated for these tests.

‡ The sera were reinactivated fifteen minutes at 56°C. for these tests.

NOTE: Neg refers to negative; tf, trace of fixation; wf, weak fixation, sf, strong fixation; cf, complete fixation.

Besides making these titrations to determine the loss of hemolysin due to heat, the writer made titrations on 97 sera before and after they had been kept for seven days in the ice box to determine the stability of anti-sheep hemolysin in human sera. Some were kept for fourteen days and again retested. Results tabulated in tables 7 and 8 seem to indicate that there is apparently no marked loss of hemolysin during this period of time. The apparent discrepancies in the titrations and the apparent increase of the hemolysin in sera nos. 4512, 4516, 4520, 4523, 4528, 4544, 32B, and 34B, after being kept for fourteen days as shown in table 8 is probably due to weak blood cells, or strong complement units, or both.

Kolmer (63) divides thermolabile and thermostable hemolysins in human sera as follows: "thermolabile hemolysins" those found in fresh unheated serum; "thermostable hemolysins" those found after the serum has been heated at 56°C. for one-half hour—he states that all these natural hemolysins with the occasional exception of anti-sheep hemolysin are completely destroyed by heating at 62°C. for one-half hour, and that immune hemolysins suffer only partial deterioration at this exposure.

Thiele and Embleton (64) state that a portion of the natural anti-sheep hemolysins present in active human sera are thermolabile, being destroyed by heating at 56°C.

Sherman (65) claims that all hemolysins are thermostable and that a reduction in hemolytic activity of a serum as a result of heating is due to "masking" of the hemolysin rather than actual destruction.

7. DETERMINATION OF THE RELATIVE ANTIGENIC VALUE OF TUBERCLE BACILLUS ANTIGENS PREPARED AND STERILIZED BY TWENTY DIFFERENT METHODS

The literature on this subject has been recently abstracted by H. R. Miller (66), and the various antigens used by the several workers tabulated in four general groups. Group 1. This group comprises those antigens composed of the whole bacillus, including not only antigens prepared from the tubercle bacilli, but also antigens prepared from other allied acid-fast bacteria. The results reported vary both in constancy and the percentage of positive fixations in positive cases. Group 2 are antigens which consist of various tuberculins. In the main the results reported by these workers have been encouraging, the chief difficulty being non-specific fixation, especially with the sera of non-tuberculous syphilitic patients. Group 3 are those antigens derived from the tubercle bacillus by means of chemical digestion and extraction. The chief workers in this field have been Deyke, Much, Leschke and Altstaedt. It is to this group that the "partial antigens" belong. Group 4 comprises antigens derived from normal and tuberculous tissue. Such antigens have not given uniform results. In going over the results of the various workers, the antigens in group 1 seemed to be the only ones that would give practical results. In the preparation of a tubercle bacillus antigen of this group, the first question which arises is whether to use a suspension of living bacilli or of killed bacilli. Corper (67) states that, while the most reliable investigators concede that a suspension of living tubercle bacilli is the only one of the many antigens used, that is of specific value, the objections to the living bacillary emulsion are the small leeway between the antigenic and the anticomplementary dose, the turbidity produced in the tubes and the fairly high percentage of non-specific reactions.

The writer has attempted on several occasions to use suspensions of living bacilli but found that they became anticomplementary very quickly and for this reason could not be used satisfactorily. Besides, the danger attending the handling of antigen made with the living bacilli precludes its general use in diagnostic laboratories. He has not, therefore, attempted to use suspensions of living bacilli in this comparative study. All his suspensions have been made from killed bacilli.

The next question which arises is the best method of killing the bacilli without destroying the antigenic value of the finished antigen.

M. A. Wilson advocated killing the bacilli in the broth culture by sterilizing in the Arnold sterilizer for one hour and then washing the bacilli from the filter paper with 95 per cent alcohol.

White of the Otisville Sanitarium advised the writer to make the antigens from the living bacilli without the use of heat, washing the living bacilli directly with alcohol and preserving all the sediment from the alcoholic mixture.

To definitely settle the above questions, the writer made up antigens in 18 different ways from one strain of tubercle bacilli grown on broth for four months and has made comparative tests with these 18 antigens, Wilson antigen no. 330 and Wilson antigen no. 400, on sera from 60 patients. Forty-five of these were tuberculosis patients and 15 were normal controls. In all 1167 comparative tests were made. The writer was unable to make comparative tests with the entire 20 antigens on all the sera as the quantity of serum obtained from some of the patients was insufficient. Duplicate comparisons were made on as many as possible. The antigens were made up as follows: The broth flask containing the tubercle bacillus growth was thoroughly shaken so that an even distribution of the tubercle bacillus pellicle was obtained. This was divided into three bottles. Bottle 1 was placed in the steam sterilizer and kept at 100°C. for one hour. It was then taken out, thoroughly shaken, and the mixture of broth and bacillus pellicle was poured on a large filter paper and allowed to drain until no more broth dripped from the lower orifice of the funnel. The filter was then broken and the sediment washed off with 95 per cent alcohol. This was left to macerate in the alcohol for ten days.

Bottle 2 was also placed in the Arnold sterilizer and heated for one hour at 100°C. This mixture was poured on a filter paper, and at once washed with cold normal saline solution until the wash water ran through perfectly clear. The filter was then broken, and the sediment was washed off the filter paper with 95 per cent alcohol and left to macerate for ten days.

Bottle 3 was not heated at all. The cold live bacillus culture was poured on a filter paper and washed with cold saline until the wash water was clear. The filter was then broken and the live bacilli were washed off the filter paper with cold 95 per cent alcohol, and left to macerate in this alcohol for ten days. At the end of ten days, the supernatant alcohol was poured off from all three bottles. The sediments of bacilli and precipitated proteins were washed in ether three or four times for a period of twenty-four hours; they were thoroughly shaken each time. The ether was poured off and the sediments were washed with ether into centrifuge tubes. These were centrifuged and the supernatant ether was poured off. In this way all constituents soluble in alcohol and ether were removed. The tubes were then loosely plugged with sterile cotton and placed in an incubator over night to dry by evaporating off the ether.

The dried powders were weighed and suspended in 0.9 per cent saline in the proportion of 1 gram of powder to 200 cc. of saline. These were thoroughly emulsified in a mortar. Suspension 1 was then divided into six bottles labelled 1A, 1B, 1C, 1D, 1E and 1F. Every precaution was observed to prevent bacterial contamination of the bacillus suspensions.

Bottle 1A was left without heating, or the addition of any preservative.

Bottle 1B was sterilized in the Arnold sterilizer at 80°C. for one hour.

Bottle 1C was sterilized in the Arnold sterilizer at 100°C. for one hour on two consecutive days.

Bottle 1D—an equal part of 95 per cent alcohol was added to the bacillus suspension, shaken and stored without heating.

Bottle 1E—one part of alcohol was added to three parts of the bacillus suspension; the mixture was shaken and stored without heating.

Bottle 1F—0.5 per cent carbolic was added to the bacillus suspension, which was stored without heating.

Suspensions 2 and 3 were similarly divided and treated as suspension 1. In this way, the writer had antigens made of the same culture, but treated in 18 different ways.

Dilutions of 1-10, 1-25 and 1-50 were made of all the A, B, C, and F antigens, and were titrated to determine their anticomplementary dose. Antigens D were made up in 2-10, 2-25, and 2-50 dilutions because these antigens were mixed with equal parts of alcohol and therefore contained only one-half as much powdered sediment as the A, B, C, and F antigens. Antigens

TABLE 9

Showing a few of the comparative tests on tuberculous and normal sera made with the antigens prepared by twenty different methods

ANTIGEN NUMBER	CLINICAL DIAGNOSIS																	
	Tuberculous														Non-tuberculous			
	Serum 1	Serum 3	Serum 4	Serum 5	Serum 7	Serum 8	Serum 11	Serum 12	Serum 13	Serum 14	Serum 31	Serum 33	Serum 40	Serum 45	Serum 51	Serum 53	Serum 92	Serum 94
1A	2+	1+	3+	3+	1+	–	4+	–	–	1+	4+	–	1+	2+	–	–	–	–
2A	1+	±	±	+	1+	–	1+	±	–	1+	4+	–	1+	1+	–	–	–	–
3A	±	1+	2+	3+	2+	–	4+	±	–	1+	4+	–	1+	1+	–	–	–	–
1B	2+	1+	2+	4+	2+	–	3+	–	–	±	4+	–	1+	2+	–	tr	tr	tr
2B	1+	–	±	±	–	–	2+	±	–	±	4+	–	1+	±	–	–	–	–
3B	2+	1+	2+	4+	–	–	4+	±	–	1+	4+	–	1+	1+	–	tr	–	–
1C	3+	1+	2+	3+	2+	–	2+	–	–	±	4+	–	1+	2+	–	–	–	tr
2C	2+	1+	±	2+	±	–	1+	–	–	±	4+	±	1+	1+	–	–	–	–
3C	3+	2+	1+	3+	3+	–	4+	–	–	1+	4+	±	1+	1+	–	–	–	tr
1D	1+	1+	1+	4+	2+	±	3+	–	–	+	4+	–	1+	1+	–	–	2+	2+
2D	–	–	–	±	±	–	±	–	–	±	4+	–	1+	–	–	–	–	–
3D	1+	3+	2+	3+	2+	–	4+	±	–	+	4+	–	2+	1+	–	tr	+	+
1E	2+	3+	2+	4+	2+	–	4+	–	–	2+	4+	–	1+	1+	–	–	±	2+
2E	1+	1+	1+	1+	±	–	2+	–	–	+	4+	–	1+	±	–	–	–	+
3E	2+	3+	2+	3+	2+	–	4+	–	–	2+	4+	–	2+	1+	–	±	+	2+
1F	2+	1+	+	2+	+	–	4+	–	–	2+	4+	–	1+	3+	–	+	–	+
2F	1+	±	–	1+	+	–	4+	–	–	2+	4+	–	1+	+	–	±	–	+
3F	2+	1+	1+	2+	2+	±	4+	–	–	2+	4+	–	1+	2+	–	±	±	2+
330	2+	1+	2+	3+	2+	–	4+	–	–	+	4+	–	1+	2+	–	–	–	–
400	2+	1+	2+	4+	2+	–	4+	–	–	+	4+	–	1+	1+	–	–	–	–

tr- refers to trace of fixation (reported as negative).

E were made up in 1.5-10, 1.5-25, 1.5-50 dilutions because in these antigens one part of alcohol was mixed with three parts of bacillus suspension and therefore contained only three-fourths as much powdered sediment as the A, B, C, and F antigens.

The titrations were set up in the following volumes: 0.05 cc., 0.1 cc., 0.2 cc., 0.3 cc., 0.4.cc., 0.5 cc., 0.6 cc., 0.7 cc. and 0.8 cc.

TABLE 10

Titration results of antigens prepared by twenty different methods for their anticomplementary activity

ANTIGEN NUMBER	QUANTITY OF ANTIGEN							
	0.1 cc.	0.2 cc.	0.3 cc.	0.4 cc.	0.5 cc.	0.6 cc.	0.7 cc.	0.8 cc.
1A	–	–	–	–	–	–	–	tr.
2A	–	–	–	–	–	–	–	tr.
3A	–	–	–	–	–	–	tr.	±
1B	–	–	–	–	–	–	–	tr.
2B	–	–	–	–	–	–	–	tr.
3B	–	–	–	–	–	–	–	tr.
1C	–	–	–	–	–	–	tr.	tr.
2C	–	–	–	–	–	–	–	tr.
3C	–	–	–	–	–	–	–	tr.
1D	–	±	+	+		+	+	+
2D	–	–	–	±		±	++	+
3D	–	–	±	+		+	++	++
1E	–	–	–	–		+	±	+
2E	–	–	–	–		±	+	+
3E	–	–	+	±		±	+	tr.
1F	–	–	–	–		–	tr.	tr.
2F	–	–	–	–		–	–	tr.
3F	–	–	–	–		–	–	tr.

All antigens were made up in 1-50 dilutions, except the D and the E antigens.

The D antigens were preserved by the addition of equal parts of 95 per cent alcohol. The final dilution was therefore made by adding 0.2 cc. of this alcohol antigen mixture to 48 cc. of 0.9 per cent saline solution.

The E antigens were preserved by the addition of 1 part of 95 per cent alcohol to 3 parts of stock antigen. The final dilution was therefore made by adding 1.5 cc. of this alcohol antigen mixture to 48.5 cc. of 0.9 per cent saline solution.

The 1-10 and the 1-25 dilutions were found to be too anticomplementary. The 1-50 dilutions gave satisfactory results as the anticomplementary dose for the A, B, and C antigens was from 0.7 to 0.8 cc. Antigens D, E, and F were anticomplementary in much lower doses, as shown in table 10.

In going over the results of the 1167 comparative tests made with these twenty antigens on the 60 sera, one notes that practically all the tests made with the no. 2 antigens gave weaker results than either the no. 1 or no. 3 antigens. Antigens 1A, 3A, 1B, 3B, 1C and 3C all gave approximately the same results, and gave practically no non-specific results with the normal control sera.

Referring to table 9 one notes that antigens D, E, and F gave stronger results on an average, but also gave a moderate number of non-specific results with the normal sera. Lower dilutions of these were tried in a few instances and more consistent results were thereby obtained. The alcohol and the carbolic acid used as preservatives apparently gave a marked anticomplementary tendency to these antigens. This was borne out by the titrations tabulated in table 10.

These observations disprove the assertion that heat destroys the antigenic value of bacillary antigens, also that antigens made from bacilli without being killed by heat are better than antigens made from heated cultures. Apparently washing the hot broth sediments on the filter paper with saline removed some of the antigenic properties of the antigens.

Bronfenbrenner (69) states that it is necessary to free each sample of tuberculin (tubercle bacillus antigen) of all its lipin fraction before using such tuberculin for the complement fixation test.

One hundred sera were tested by a worker in this laboratory with an antigen made from bovine tubercle bacilli. There was no distinction with the bovine and human antigens. The variations were in about the same proportion as occurred in tests with some of our different kinds of human strain antigens. One hundred sera were tested with the glycerine extract antigen sent to the laboratory by Petroff (68). This antigen gave the same reaction as M. A. Wilson's in 95 per cent of the cases, and in 5 per cent the reaction was weaker with Petroff's antigen.

8. DETERMINATION OF THE RELATIVE VALUE OF THE HECHT-GRADWOHL TECHNIC AND THE TECHNIC OF NATURAL ANTI-SHEEP AMBOCEPTOR ABSORPTION IN COMPARISON WITH THE STANDARD COMPLEMENT FIXATION TEST

The Hecht-Gradwohl tests were performed with the simplified technic advocated by W. J. Bruce (70).

According to Lewis and Newcomer (71) every one of 70 consecutive fresh, sera contained enough native complement for a Hecht-Gradwohl test if susceptible corpuscles were used.

Gradwohl (72) concluded that the Hecht-Gradwohl test in his hands has been a far better check or control of the Wassermann reaction than the use of any of the controls now in vogue. The so-called border-line Wassermanns if truly positive will show a strong Hecht-Gradwohl reaction. In other words, the Hecht-Gradwohl is a far more sensitive test than the original Wassermann reaction.

In his mind, no negative Wassermann was worth anything unless backed up by a Hecht test. He stated that even with this control test 25 per cent of syphilitic sera will give negative reactions.

L. E. Schmidt of Chicago, Carl C. Warden of Ann Arbor, M. L. Heidengspeld of Cincinnati and others concluded that this test was a very good control to use with the Wassermann reaction but that it should not be used alone as it may be too sensitive.

Rubinstein (73) and Radossavlievitch (74) stated that the results of the Hecht system did not offer as much guarantee of specificity as the standard technic.

Eschbach and Duhot (75) guarded against the inevitable danger of doubtful reactions, which are provoked with the Hecht method, by the use of a very dilute antigen.

In this study, the natural antisheep amboceptor was removed from the sera by the addition of equal parts of a 10 per cent sheep cell suspension. This mixture was incubated for one hour, centrifuged and the clear serum pipetted off from the sedimented blood cells. As the serum was diluted 1 to 2, two times the regular volume was used in setting up the tests. This is the method advocated by Bauer (76–77). The procedure sometimes causes the sera to become anticomplementary, which is known as the Sachs-Friedberger phenomenon.

Rossi (78) advised absorbing the heated serum at a low temperature—keeping the serum-blood-cell mixture packed in ice and chilled during the centrifuging.

Wechselmann (80) advocates absorption of hemolysin from sera with barium sulphate. Noguchi and Bronfenbrenner (81) found that this procedure removed the natural antisheep hemolysin and also syphilitic antibody.

Simon (79) has also described a method for removing the natural antisheep amboceptor. For ten minutes 0.4 cc. of sera is inactivated at 56°C., mixed with 1.6 cc. of 2.5 per cent sheep cell suspension, extracted for ten minutes at 37°C. in a water bath and centrifuged free from corpuscles; each cubic centimeter then contains 0.2 cc. of serum.

Jacobaens (82) found in a study of 257 cases, 10 per cent more positive Wassermann reactions after absorbing the sera with sheep corpuscles. Olmstead (83), Van Saun (84), Ottenberg (85), Sielman (86), Stern (87), Kaliski and others have advocated various methods for the closer adjustment of the antisheep hemolytic system to avoid the effect of excessive amounts of natural hemolysin.

Stimson (88) stated that the criticism of the use of sheep cells in complement fixation tests on the ground of their being subject to the action of native amboceptor in human sera is not altogether well founded. It is granted that a fair percentage of human sera have this amboceptor, but these same sera may give excellent fixation, because if the complement has been fixed in the first phase, the mere increment of amboceptor will not cause hemolysis in the second phase. However, the removal of natural antisheep amboceptor will somewhat increase the percentage of positive reactions in a series of tests.

Neill (89) found in a series of experiments that while the natural antisheep hemolysin does reduce fixation when present in syphilitic sera, the amount of hemolysin must be very large and the amount of syphilitic antibody very small in order to produce a significant effect. He concludes that the presence of antisheep hemolysin in sera is not an objection to the use of the sheep cell hemolytic system, if the sera are employed in amounts corresponding to not less than 0.1 cc. to a total of 5 cc. for the test.

Kolmer found that with sera containing large amounts of syphilitic antibodies excessive amounts of hemolysin have practically no influence upon the results or, at the most, reduce a 4 plus to a 3 plus reaction; but weak positive results are often reduced to negatives when a large excess of hemolysin is present if the readings are made after the tubes have stood in the refrigerator over night. Readings made immediately after taking from the water bath did not show such marked differences.

However, all methods of removing the natural antisheep amboceptor are probably too time consuming for a routine diagnostic laboratory to perform when large numbers of sera are examined each day.

The writer has made comparative tests on sera from 60 patients, forty tuberculous individuals and twenty normal controls. In these comparisons the same antigen (C3—1-50 dilution, 0.2 cc.) and the same sheep cells were used for all the tests.

In table 11 thirty-five of these comparative tests have been tabulated. The tests were run in four sections: first, the standard complement fixation test was made on the serum one day after its removal from the patient; second, the Hecht-Gradwohl modification was performed on the serum two days after its removal from the patient; third, the serum was tested after the natural antisheep amboceptor had been absorbed from it; fourth, the standard complement fixation test was again made on the serum after keeping it for seven days in the ice chest under sterile precautions.

Upon examining the chart, one notes that the tests agreed in the majority of cases. However, there are a moderate number of variations. By absorbing out the natural antisheep amboceptor, 5 normal sera gave some degree of fixation; 5 tuberculous sera gave stronger fixation results than the first day standard test; 3 tuberculous sera, negative with the standard test, gave

TABLE 11

Showing a few comparative tests made with the regular complement fixation test on the first and seventh days, the Hecht-Gradwohl test and after absorption of the natural antisheep amboceptor

SERUM NUMBER	REGULAR TEST, FIRST DAY*	HECHT-GRADWOHL TEST†	AFTER ABSORPTION OF NATURAL ANTISHEEP AMBOCEPTOR†	REGULAR TEST, SEVENTH DAY*	CLINICAL DIAGNOSIS
1	±	1+	neg	1+	Normal
2	neg	neg	neg	±	Tuberculosis type IV
3	neg	neg	neg	neg	Normal
4	neg	neg	neg	neg	Moderately advanced, tuberculosis type I
5	neg	neg	neg	neg	Normal
6	4+	4+	4+	4+	Moderately advanced, tuberculosis type III
7	neg	+	neg	neg	Suspicious
8	1+	1+	1+	1+	Moderately advanced, tuberculosis type III
9	neg	neg	neg	±	Moderately advanced, tuberculosis type IV
10	1+	neg	neg	1+	Far advanced, tuberculosis type IV
11	neg	neg	neg	neg	Normal
12	neg	±	neg	1+	Moderately advanced, tuberculosis type IV
14	2+	2+	1+	2+	Moderately advanced, tuberculosis type III
15	neg	±	neg	neg	Normal
16	4+	2+	4+	4+	Tuberculosis type III
17	neg	+	±	±	Tuberculosis type II
18	neg	neg	neg	neg	Tuberculosis type I
19	3+	4+	3+	4+	Tuberculosis type III
20	4+	4+	4+	4+	Tuberculosis type III
21	tr	neg	+	+	Tuberculosis type III
22	1+	neg	2+	+	Tuberculosis type III
23	1+	4+	+	+	Tuberculosis type II
31	4+	4+	4+	4+	Incipient, tuberculosis type II
32	neg	neg	2+	+	Incipient, tuberculosis type II
33	neg	4+	neg	neg	Incipient, tuberculosis type II
35	neg	+	4+	+	Incipient, tuberculosis type II
37	1+	4+	3+	2+	Incipient, tuberculosis type II
40	±	ac	+	±	Incipient, tuberculosis type I
41	neg	2+	±	neg	Incipient, tuberculosis type I
42	4+	+	4+	4+	Incipient, tuberculosis type II
44	neg	+	2+	neg	Incipient, tuberculosis type II
52	neg	neg	±	tr	Normal
53	neg	neg	+	neg	Normal
58	neg	+	2+	tr	Normal
60	neg	neg	2+	+	Normal

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* Refers to the day the reaction was made after the specimen was removed from the patient.

† These tests were made two days after the specimens were removed from the patients.

NOTE: neg = negative; tr = trace; ac = anticomplementary.

positive results with the Hecht-Gradwohl modification; 6 tuberculous sera, negative with the standard test, gave positive results and 2 tuberculous sera, positive with the standard test, gave negative results with the amboceptor absorption method.

In reviewing the results, one is struck by the fact that some sera from tuberculous patients will not fix complement with any of the modifications tried out, even when there is a certain amount of non-specific fixation going on in the normal control sera due to the weak system used. Also, as soon as one attempts to make the complement fixation test more sensitive, normal sera begin to give some degree of fixation. While both the Hecht and the amboceptor absorption methods gave more positive results with the sera from tuberculous patients, they also gave some non-specific results with normal sera.

9. COMPARATIVE RESULTS OBTAINED BY TESTS WITH SERA ONE DAY OLD AND WITH THESE SAME STERILE SERA AFTER PRESERVING FOR ONE WEEK IN THE ICE BOX

These comparative tests have been made on sera from 900 patients (500 tuberculous and 400 non-tuberculous). In a previous publication by the writer, he reported that certain sera which gave negative or weak positive reactions when tested on the day after the specimens were taken from the patient, gave strong positive reactions when tested after the specimens had been kept for one week under sterile precautions in the ice chest. At that time, he stated that this occurred in a large percentage of cases and was due, he thought, to the presence of some inhibitory substance in the serum which disappeared after the week's interval. He also stated that he did not observe this phenomenon in any of the sera from non-tuberculous cases.

While we have continued to observe this difference in fresh and kept sera (note table 12) it has been in a much lower percentage of tuberculous cases and has also appeared in a moderate number of non-tuberculous cases, showing that it is not a specific phenomenon and that it is probably caused by certain anti-

TABLE 12

Results of complement fixation tests made on the first and seventh days

SERUM NUMBER	FIRST[*]	SEVENTH[*]	DIAGNOSIS	SERUM NUMBER	FIRST	SEVENTH	DIAGNOSIS
1	2+	2+	Tuberculous	31	4+	4+	Tuberculous
2	4+	4+	Tuberculous	32	−	+	Tuberculous
3	2+	+	Tuberculous	33	−	−	Tuberculous
4	3+	3+	Tuberculous	34	−	−	Tuberculous
5	4+	4+	Tuberculous	35	tr	+	Tuberculous
6	±	±	Tuberculous	36	−	−	Tuberculous
7	4+	4+	Tuberculous	37	+	2+	Tuberculous
8	−	−	Tuberculous	38	−	−	Tuberculous
9	−	−	Tuberculous	39	−	−	Tuberculous
10	−	−	Tuberculous	40	−	±	Tuberculous
11	4+	4+	Tuberculous	41	−	−	Tuberculous
12	−	±	Tuberculous	42	4+	4+	Tuberculous
13	−	−	Tuberculous	43	−	−	Tuberculous
14	3+	4+	Tuberculous	44	tr	−	Tuberculous
15	4+	4+	Tuberculous	45	2+	3+	Tuberculous
16	tr†	+	Tuberculous	51	−	−	Non-tuberculous
17	+	+	Tuberculous	52	−	+	Non-tuberculous
18	+	+	Tuberculous	53	−	tr	Non-tuberculous
19	−	−	Tuberculous	54	−	−	Non-tuberculous
20	tr	−	Tuberculous	55	−	tr	Non-tuberculous
21	−	−	Tuberculous	56	+	±	Non-tuberculous
22	−	−	Tuberculous	57	±	3+	Non-tuberculous
23	−	−	Tuberculous	58	−	−	Non-tuberculous
24	−	−	Tuberculous	59	±	±	Non-tuberculous
60	−	+	Non-tuberculous	148	−	−	Non-tuberculous
61	4+	3+	Tuberculous	149	−	−	Non-tuberculous
62	+	−	Tuberculous	150	−	−	Non-tuberculous
126	−	−	Non-tuberculous	151	−	−	Non-tuberculous
127	−	−	Non-tuberculous	152	+	+	Tuberculous
128	−	−	Non-tuberculous	153	2+	4+	Tuberculous
129	−	−	Non-tuberculous	154	+	+	Tuberculous
130	−	−	Non-tuberculous	155	−	−	Non-tuberculous
131	−	−	Non-tuberculous	156	4+	4+	Tuberculous
132	−	−	Non-tuberculous	157	tr	+	Tuberculous
133	−	−	Non-tuberculous	158	−	−	Tuberculous
134	−	−	Non-tuberculous	159	±	4+	Tuberculous
135	−	−	Non-tuberculous	160	−	−	Tuberculous
136	−	−	Non-tuberculous	161	±	+	Tuberculous
137	−	−	Non-tuberculous	162	+	2+	Tuberculous
138	−	−	Non-tuberculous	163	−	−	Tuberculous

TABLE 12—*Concluded*

SERUM NUMBER	FIRST*	SEVENTH*	DIAGNOSIS	SERUM NUMBER	FIRST	SEVENTH	DIAGNOSIS
139	–	–	Non-tuberculous	164	–	4+	Tuberculous
140	–	–	Non-tuberculous	165	–	–	Tuberculous
141	–	–	Non-tuberculous	166	–	–	Tuberculous
142	–	–	Non-tuberculous	167	2+	4+	Tuberculous
143	–	–	Non-tuberculous	168	+	+	Tuberculous
144	–	–	Non-tuberculous	169	+	–	Tuberculous
				171	–	–	Non-tuberculous
145	–	–	Non-tuberculous	172	–	–	Tuberculous
				173	–	–	Tuberculous
146	–	2+	Non-tuberculous	174	–	–	Non-tuberculous
				175	–	–	Non-tuberculous
147	–	–	Tuberculous	176	–	–	Non-tuberculous

* Day reaction was made after the specimen was taken from the patient.
† tr = trace of fixation.

complementary changes, loss of natural antisheep amboceptor or by the unavoidable differences in the complement, blood cells, and antigen suspensions used in these comparative tests.

The fact that in my previous publication a high percentage of negative or weak positive results was reported with tuberculous sera on the first day tests which on the seventh day gave markedly stronger reactions, and that no non-tuberculous sera showed this change, was probably due to one or more of the following reasons: First, the antigen used in this series had a very wide range between its anticomplementary unit and its antigenic unit, and only two antigenic units were used: i.e., 0.1 cc. of a 1-50 dilution. Second, only the classical Wassermann volumes of sera were used while in all our later tests twice the regular volumes of sera had been used as a basis for our reports. Third, nearly all the tuberculous cases in this series showing this marked change were active type 2 cases which should have given strong positive reactions on the first day tests. The antigen dose was probably too weak to bring this out. The additional help of slight anticomplementary changes, the loss of some of the natural antisheep amboceptor due to the extra fifteen minute inactivation, and other unknown and uncontrollable factors when added to the specific tubercular reaction were sufficient to give specific positive results but not sufficient to give positive reactions with the normal sera.

In the last series of 101 cases every precaution was taken to avoid or to note any difference between the antigen, complement and blood cells used on the first and the seventh day tests.

The complement used was carefully titrated against sheep cells and then salted (by the addition of 9 per cent dry powdered salt to the concentrated complement). This salted complement and a portion of the unwashed sheep cells was then packed in ice in a thermos bottle and kept in this way for the seven-day period. Even after these precautions, differences were found in the antigenic units on the first day and the seventh day tests on several occasions.

In summing up the results of the first and seventh day tests on sera from a group of 101 patients (61 tuberculous individuals and 40 normal controls), 15 tubercular sera gave stronger reactions on the seventh day than on the first day tests, 4 of these changed from complete negative to positive. At the same time, 4 of the 40 normal control sera changed from negative or doubtful reactions to some degree of positive ranging from plus-minus to positive 3 plus. These results are shown in table 12.

It, therefore, seems probable that no change in the specific fixability of the tuberculous sera occurs, but that the apparent change is due to anticomplementary changes and non-specific variations in the reagents employed in making the tests.

Rapoport in 1919 (90) made a similar observation during his study of the fixation test in influenzal pneumonia, using strains of *B. influenzae* as antigen, stating that the fixation test on sera made during the time the patient was still acutely ill were in many cases negative or slightly positive; but when these sera were kept in the refrigerator for from six days to three weeks, many gave strong positive reactions.

10. DETERMINING WHETHER ANY CROSS FIXATION EXISTS BETWEEN THE TUBERCLE BACILLUS ANTIGENS USED IN THIS STUDY AND SYPHILITIC ANTIBODIES

During this study the writer has made Wassermann reactions, as well as complement fixation reactions for tuberculosis on the sera from 192 patients to determine the possibilities of non-specific cross fixation with sera giving strong Wassermann reactions.

Eighty-five of these sera gave positive Wassermann reactions and absolutely negative tuberculosis complement fixation reactions; 104 gave negative reactions with both the Wassermann and the tubercle bacillus antigens; 2 gave positive reactions with both antigens and had clinical histories and symptoms of both diseases; and 1 gave positive results with both antigens whose history could not be obtained. This test was made at a general army hospital, and as the writer was shortly afterwards transferred to another camp, he was unable to follow up the case and determine whether or not the patient had tuberculous complications.

At any rate, 85 out of 85 positive Wassermann sera gave negative tuberculosis complement fixation tests.

Burns (91) of the Boston Board of Health reported that he made 912 complement fixation tests on sera which they received for routine Wassermann tests. Of these 221 were positive and 691 were negative for the Wassermann reaction.

Of the 221 positive Wassermann tests, complement fixation tests for tuberculosis gave 21 positive, 29 doubtful, and 171 straight negative. Of the 691 negative Wassermann tests, 55 reacted positively for tuberculosis, 35 moderately positive. There were 21 delayed negative and 580 negative reactions.

They were unable to obtain clinical histories in many of these cases to confirm the specificity of the tuberculous reactions; but the evidence seemed conclusive that there was no cross-fixation with syphilitic serum.

The percentage of positives, 15, seems higher than should be expected; but when we consider that tuberculosis causes the death of about one person in every ten and that careful observers calculate at least three living active cases to each death, perhaps this percentage is not excessive, especially among that class of patients who appear for free Wassermann tests.

From their observations, it appeared that the test was specific for tuberculosis.

Bronfenbrenner (92) stated that the complement fixation test for tuberculosis, using the Besredka antigen, appeared to be specific for tuberculosis.

Craig stated that in his experience the complement fixation test with his tubercle bacillus antigen does not give positive results with syphilitic sera when no coincident tubercular infection is present. He also concluded that a positive reaction is specific and that it apparently indicates active lesions.

H. J. Corper (93) is one of the few serologists who do not agree with the above statements of the high percentage of specific results with this test. He stated that in his series of cases, he used 92 sera with positive Wassermann reactions and of these, 65 gave cross fixation with all tubercle bacterial antigens. He, therefore, concluded that in the presence of a positive Wassermann reaction, the presence of a positive complement fixation test for tuberculosis is of no practical value.

11. THE RELATIONSHIP OF THE VON PIRQUET REACTION TO THE COMPLEMENT FIXATION TEST FOR TUBERCULOSIS

In this study the writer has been able to compare the results of the von Pirquet reaction with the complement fixation test on only a small number of cases, as both the clinicians and the patients objected to the von Pirquet test.

However, when the results of this small number of comparisons are examined on table 13 we at once see that no parallel relationship exists between these two reactions.

Boez (94) states that the reaction of fixation has no necessary relation of coexistence or intensity with the cutaneous reaction. During the first stage of pulmonary tuberculosis the cutaneous reaction and reaction of fixation generally exist together. The dissociation following the type where the cutaneous reaction is negative, and the fixation reaction is positive indicates a step advanced and an evolution unfavorable to the patient. Finally, the formula is modified to the terminal phase; failure of all of the reactions, even the fixation reactions disappearing.

TABLE 13

Table showing the comparison between the von Pirquet reaction and the complement fixation test

SPECIMENS FROM PATIENTS WITH POSITIVE VON PIRQUET REACTIONS		SPECIMENS FROM PATIENTS WITH NEGATIVE VON PIRQUET REACTIONS	
Positive fixation	Negative fixation	Positive fixation	Negative fixation
23* 74 per cent	8 26 per cent	8 61 per cent	5 39 per cent

* Refers to the number of patients.

12. THE CLASSIFICATION OF TUBERCULOSIS PATIENTS

The classification of pulmonary tuberculosis has been undertaken by many investigators since ancient times. Noted classifications have been made by Williams of the Brompton Hospital, Cornet, L. Bard, Koeniger, Turban (95–96), Meissen (97) and Walter Rathbun (98). Rathbun classifies tuberculosis as incipient A, B, and C; moderately advanced A, B, and C; far advanced A, B, C.

While Rathbun's classification, accepted by the American Sanatorium Association, undoubtedly is a very broad classification and takes in every possible stage of the disease, it is divided into so many divisions (nine) that no two clinicians can ever agree on the types in a group of 100 cases. Also, this classification, if condensed into its three main divisions only; i.e., incipient, moderately advanced and far advanced, disregarding the activity of the disease manifested by the patient at any given time, does not run parallel with the degree of antibody production in the patient's blood serum. As the fundamental basis of the complement fixation test is the degree of antibody production in the patient's blood serum, and as this antibody production is primarily based on the activity of the disease regardless of whether it is incipient or far advanced, no parallel relationship can exist between this classification and the test under investigation.

In this study the complete clinical data has been obtained on practically all of the cases, and X-ray findings, von Pirquet tests and Wassermann reactions have been made on a large number. The clinical data consists of age, past and present temperature, pulse and respiration records, sputum reports and physical symptoms with the clinical diagnoses and classifications. The classifications were made by various diagnosticians, who used the National Association's, Stoll's and a new classification by the writer. Many of the recent investigators agree that their highest percentage of positive fixations are in the moderately advanced cases, while the far advanced cases give a rather low percentage of positive results. The cases with a very poor prognosis, especially the laryngeal cases frequently give absolutely negative results.

In checking up the findings of all the series of tests by means of the National Association's classification, the results were rather indefinite. One particularly noticed that the positive findings were most numerous and most definite in the cases showing active constitutional symptoms regardless of whether they were incipient, moderately advanced or far advanced; and, vice versa, the positive results were least numerous and frequently doubtful in the cases where there were slight or no constitutional symptoms again regardless of the degree or stage of the lesions. In fact, the strength and percentage of the positive findings ran a fairly parallel course with the degree of activity manifested by the patient's constitutional symptoms rather than by the stage of the disease or the extent of the lesion.

The early or incipient cases even with very small lesions but with active constitutional symptoms gave quite a high percentage of negative results.

The writer, has, therefore, arranged a classification based on the antibody production in the patient's blood serum as indicated by the patient's constitutional symptoms. Incipient A, moderately advanced A, and far advanced A cases produce relatively few antibodies. Incipient B, and incipient C produce more, moderately advanced B and far advanced B apparently produce the most antibodies in the greatest percentage of cases. Moderately advanced C and far advanced C frequently are overwhelmed by the disease and therefore produce only few or no detectable antibodies.

To be of any value to the clinician, a classification of this kind must be interchangeable with those most commonly used by the clinicians. The following simple classification, while by no means perfect, fits in fairly well with the complement fixation findings and can easily be transcribed by the clinician in terms of the National Association's or Stoll's classifications.

THE WRITER'S CLASSIFICATION	THE NATIONAL ASSOCIATION CLASSIFICATION	STOLL'S CLASSIFICATION
Non-tubercular patients	Negative controls	V1
Suspects	Suspects	5A and 5B
Inactive	Inactive cases	Inactive
Type I	Incipient A, moderately advanced A, and far advanced A	1A, 1B, 2A, and 3A
Type II	Incipient B, incipient C	1C and 2B
Type III	Moderately advanced B and far advanced B	3B
Type IV	Moderately advanced C, and far advanced C	1V

The following classification was published by the writer in a previous communication (99).

Type I. Primary cases; very few physical symptoms present; no tubercle bacilli found in the sputum or found only after the examination of many specimens by the antiformin method.

Type II. Active cases; patient expectorating tubercle bacilli, the diseased area being walled off incompletely or not at all.

Type III. Active cases in the last stage; patient in a dying condition.

Type IV. Partially inactive cases; that is, cases expectorating tubercle bacilli but having very marked fibrous formation with the consequent complete walling off of the diseased area from the body proper.

Type V. Inactive cases.

Type VI. Cases reported as suspicious but expectorating no tubercle bacilli and having no symptoms of tuberculosis.

Type VII. Non-tubercular cases.

As this classification was not interchangeable with those commonly used by the clinicians it was dropped.

The writer has tried definitely to answer the question, "Which type of tuberculosis gives the most and strongest positive results?"

With this idea in view, he attempted to obtain a series of cases in which there would be no question as to the accuracyof the classification of the patients. He personally took all the specimens from the patients in two duplicate series, three weeks apart. The physician in charge assured him that the classifications (made by the National Association's grouping) would be as accurate as they could be made. When the tests were all completed and the classifications of the first set were compared with the second set on the same patients made three weeks later, a wide discrepancy in the duplicate classifications made by the hospital physicians was found. This was especially noticeable on the female side where a difference of 50 per cent in the comparison of the first and the second classifications was found. The male side showed a difference of 21 per cent in this comparison. This shows the extreme difficulty of correctly classifying the various types of tuberculosis patients by a classification as broad as the National Association's, and also how difficult it is for the serologist correctly to tabulate his data according to the types of cases.

Mourseend (100) said in his recent paper on the complement fixation test for tuberculosis that no effort would be made to give a detailed classification of the cases as it was felt that in the hands of different clinicians the same set of cases would receive different classifications. Cases considered as incipient by some observers would be considered as moderately advanced by others, and *vice versa*.

13. THE RELATIONSHIP OF THE PATIENT'S TEMPERATURE, PULSE, RESPIRATION AND AGE TO THE COMPLEMENT FIXATION TEST

In attempting to determine whether the temperature, pulse and respiration records of the patients bore any direct relationship to the complement fixation reactions, the writer has compared his laboratory findings with the record charts, and he has found that the cases giving a 4 plus reaction had an average temperature of 99.6; pulse, 104; and respiration, 29. The patients giving a 2 plus reaction had an average temperature of 99; pulse, 94; respiration, 25. Those giving a doubtful or negative reaction had an average temperature of 99; pulse, 88; and respiration 23. Fifty-five per cent of the 4 plus cases and only 10 per cent of the doubtful and negative cases had a temperature of over a hundred.

These averages are somewhat misleading as there were enough wide discrepancies in all the groups to bring the averages rather close together. However, a large percentage of cases having high temperature, pulse and respiration records gave strong positive reactions; the percentage of positive results running about parallel with the temperature, pulse or respiration record; i.e., the lower the temperature, pulse or respiration, the fewer were the positive results.

Sixty-five per cent of the 4 plus, 12 per cent of the 2 plus and only 5 per cent of the doubtful and negative cases had a respiration record of 30 or over. The writer also attempted to see whether, possibly, the age of the patient had any effect on his reactions and found that the average age of the patients giving a 4 plus reaction was 38; the average age of those giving a 2 plus reaction was 35; the average age of those giving a 1 plus reaction was 40; the average age of those giving a plus-minus reaction was 34. As all of the groups contained patients both young and old, no conclusions could be drawn.

14. SUMMARY OF SIX SERIES OF COMPLEMENT FIXATION TESTS

These tests were made in six separate series. The first during the winter of 1917–18 on 200 specimens of blood serum collected from the Westchester County Hospital and the New Rochelle Hospital. One hundred and one of these were from tuberculous cases and 99 from patients suffering from various ailments in the general wards. The second series was performed in the spring of 1918 on 154 specimens of blood serum obtained from patients in three general hospitals, two tuberculosis hospitals and a tuberculosis sanitarium in Westchester County, New York.

The third series was performed at the Walter Reed General Army Hospital in Washington, D. C., during the summer of 1918 on 187 specimens of blood serum from patients in the tuberculosis wards and the syphilis wards of this hospital.

The fourth series was performed during the winter of 1918, on 168 specimens of blood serum from patients in Bellevue Hospital in New York City and three general hospitals in Westchester County, New York.

TABLE 14

Report of complete study embracing all six series

Number of complement fixation tests made	6128
Number of sera examined	1207
Number of patients examined	1000
Number of clinically tuberculous patients	484
Number of non-tuberculous patients	516
Number of all types of active cases giving + reaction	331 or 69.8 per cent
Number of all types of active cases giving ± or − reaction	157 or 30.2 per cent

NUMBER	TYPE*	POSITIVE	DOUBTFUL OR NEGATIVE
539	Non-tuberculous	9 or 1.7 per cent	530 or 98.3 per cent
71	Suspects	11 or 15.5 per cent	71 or 84.5 per cent
47	Inactives	6 or 12.7 per cent	41 or 87.3 per cent
135	1	55 or 40.8 per cent	80 or 59.2 per cent
52	2	36 or 69.2 per cent	16 or 30.8 per cent
243	3	207 or 85.2 per cent	36 or 14.8 per cent
58	4	33 or 57.0 per cent	25 or 43.0 per cent

* Refers to the writer's classification.

The fifth series was a special series of 318 specimens of serum; 196 of these were from the New York City Tuberculosis Sanitarium at Otisville, New York; the remaining 122 were negative and positive controls from the general hospitals in Westchester County. In this series, the writer tried to obtain serum from 100 tuberculous patients and 75 non-tuberculous patients on two different occasions three weeks apart. The duplicate specimens were taken in order to check the accuracy of the first run of tests. He failed to obtain the duplicate specimens from a moderate number of the patients as they either refused or had left the hospitals.

The first lot of 106 specimens from the tuberculous cases of this fifth series was tested on April 24, and again on May 1. At this time, only the names of the patients and the numbers of the specimens were recorded, as we did not wish to have the histories or clinical data influence the results. The second lot of 93 specimens was taken on May 15 from most of the tuberculosis patients mentioned in the first group. In this second group, only the numbers running from one to 93 were known, but not the names, and of course, no clinical data; so again the laboratory findings could not be influenced by the personal equation. The sera from the non-tuberculous cases were tested with both groups. The results of these two groups agreed in the majority of cases.

TABLE 15

Showing the percentages of positive results obtained in the different types of cases in the six series of complement fixation tests

TYPE OF CASE	SERIES 1	SERIES 2	SERIES 3	SERIES 4	SERIES 5	SERIES 6
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Normals (non-tuberculous)	0	2	3	3	3	1.4
Suspects	0	12	20	0	25	33.0
Inactives	25	0	0	0	No cases	21.0
Tuberculosis type I	31	28	30	20	60	23.0
Tuberculosis type II	100	100	60	No cases	60	73.0
Tuberculosis type III	98	84	92	87	84	90.0
Tuberculosis type IV	27	0	0	59	80	60.0

The sixth series is the combined work of all the special antigen tests, Hecht-Gradwohl comparisons and odd tests not included in the other series. The results of the tests in these six series are given in table 14.

The percentages tabulated on table 15 are the positive results of all the tests made on the sera in each group and from each type of case.

Table 16, series 1, gave 100 per cent positive results with the type II patients (these are the incipient cases with some con-

TABLE 16

Report of the First series

Number of complement fixation tests made	1127
Number of sera examined	200
Number of patients examined	160
Number of clinically tuberculous patients	70
Number of clinically non-tuberculous patients	90
Number of Type 1, 2, 3, and 4 cases giving + reactions	61 or 73 per cent
Number of Type 1, 2, 3, and 4 cases giving ± or negative reactions	22 or 27 per cent

TYPE OF CASE	NUMBER IN EACH TYPE	NUMBER OF POSITIVE RESULTS	NUMBER OF DOUBTFUL RESULTS	PERCENTAGE OF POSITIVE RESULTS	PERCENTAGE OF DOUBTFUL RESULTS	PERCENTAGE OF POSITIVE RESULTS WHICH WERE 3+ or 4+
Non-tuberculous*	99	0	0	0	0	0
Suspects	4	0	0	0	0	0
Inactives	12	3	0	25	0	0
I	19	6	5	31	26	0
II	6	6	0	100	0	83
III	47†	46	1	98	2	90
IV	11	3	3	27	27	100

* Refers to patients in the general wards of hospitals suffering from various diseases and diagnosed as clinically non-tuberculous.

† Two sera anticomplementary.

TABLE 17

Report of second series

Number of complement fixation tests made	1458
Number of sera examined	154
Number of patients examined	154
Number of clinically tuberculous patients	70
Number of clinically non-tuberculous patients	84
Number of Type 1, 2, 3 and 4 cases giving + reactions	39 or 68 per cent
Number of Type 1, 2, 3 and 4 cases giving ± or negative reactions	18 or 32 per cent

NUMBER	TYPE	POSITIVE		DOUBTFUL		NEGATIVE	
		Number	Per cent	Number	Per cent	Number	Per cent
60	Non-tuberculous	1	2	3	5	56	93
23	Suspects	2	12	5	20	16	68
9	Inactives	0	0	3	33	6	66
14	I	4	28	5	36	5	36
4	II	4	100	0	0	0	0
37	III	31	84	4	10	2	6
2	IV	0	0	1	50	1	50
5ac.							

stitutional symptoms) and 98 per cent positive results with the type III patients (these are the moderately and far advanced cases in good condition but with constitutional symptoms).

Table 17, series 2, gave 100 per cent fixations in the type II, and 84 per cent in the type III cases.

TABLE 18

Report of third series

Number of complement fixation tests made	634
Number of sera examined	187
Number of patients examined	160
Number of clinically tuberculous patients	77
Number of clinically non-tuberculous patients	83
+Wassermann sera giving + T. B. results	1
+Wassermann sera giving − T. B. results	40
−Wassermann sera giving + T. B. results	1
−Wassermann sera giving − T. B. results	37
Number of Type 1, 2, 3 and 4 cases giving + reactions	33 or 67 per cent
Number of Type 1, 2, 3 and 4 cases giving ± or − reactions	16 or 33 per cent

NUMBER OF CASES	TYPE	POSITIVE		DOUBTFUL AND NEGATIVE	
		Number	Per cent	Number	Per cent
84	Non-tuberculous	2	3	82	97
30	Suspects	6	20	24	80
11	Inactives	0	0	11	100
13	I	4	30	9	70
10	II	6	60	4	20
25	III	23	92	2	8
1	IV	0	0	1	100
13	Unclassified and contaminated				

Table 18, series 3, gave 60 per cent fixations in the type II, and 92 per cent in the type III cases.

Table 19, series 4, included no type II cases, and gave 87 per cent positive results with the type III cases.

Table 20, series 5, gave 60 per cent positive results in type I, 60 per cent in type II, 84 per cent in type III and 80 per cent in type IV cases.

Table 21, series 6, gave 73 per cent positive results in the type II, 90 per cent in type III and 60 per cent in type IV cases.

Table 14. All the types of tuberculosis patients in these six series including incipient, moderately advanced and far advanced cases regardless of whether they were showing constitutional symptoms or not, gave 69.8 per cent positive results; the sera from the non-tuberculous controls gave 1.7 per cent positive results on separate single examinations. Repeated examinations of some of the sera from the non-tuberculous cases which gave positive reactions on one or more of the tests were made. These sera on repeated examinations did not continue to give positive fixation, showing that the positive fixations were probably non-specific due possibly to anticomplementary bodies combining with the antigen and complement.

TABLE 19

Report of the fourth series

Number of complement fixation tests made	814
Number of sera examined	168
Number of patients examined	140
Number of clinically tuberculous patients	80
Number of clinically non-tuberculous patients	60
Number of Type 1, 2, 3 and 4 cases giving + reactions	32 or 62 per cent
Number of Type 1, 2, 3, and 4 cases giving ± or − reactions	20 or 38 per cent

NUMBER OF CASES	TYPE	POSITIVE		DOUBTFUL AND NEGATIVE	
		Number	Per cent	Number	Per cent
72	Non-tuberculous	2	3	70	97
4	Suspects	0	0	4	100
1	Inactives	0	0	1	100
14	I	3	20	11	80
0	II	0	0	0	0
24	III	21	87	3	13
14	IV	8	59	6	41
39	Unclassified*	24	62	15	38

* Refers to clinically tuberculous patients whose history and classification the writer was unable to obtain.

In the report of the first series, 73 per cent of all types of active tuberculous cases gave positive reactions. The second series gave 68 per cent positive reactions, the third series 67 per cent, the fourth series 62 per cent, the fifth series 73 per cent, and the sixth series 73.2 per cent. The average percentage of positive results on complement fixation tests, embracing all the work up to date, and from all types of active tuberculosis patients is 69.8 per cent.

TABLE 20A

Report of fifth series (Otisville series)

Number of complement fixation tests made	579
Number of sera examined	268
Number of patients examined	185
Number of clinically tuberculous patients	126
Number of clinically non-tuberculous patients	59

TYPE	FIRST SPECIMENS WITH NUMBER 170 ANTIGEN		SECOND SPECIMENS WITH NO. 170 ANTIGEN		FIRST SPECIMENS WITH NO. 319 ANTIGEN		SECOND SPECIMENS WITH NO. 319 ANTIGEN		FIRST SPECIMENS WITH NO. 125 ANTIGEN	
	+	−	+	−	+	−	+	−	+	−
Non-tuberculous....	1 2%	46 98%	1 4%	22 96%	0	47 100%	2 9%	21 91%	0	40 100%
Suspects...	1 25%	3 75%	0	0	0	4 100%	0	0	2 50%	2 50%
I........	16 47%	18 53%	20 63%	12 37%	16 50%	16 50%	13 40%	19 60%	20 60%	13 40%
II........	6 54%	5 46%	6 60%	4 40%	3 28%	8 72%	3 30%	7 70%	6 54%	5 46%
III........	30 70%	14 30%	28 80%	7 20%	24 56%	19 44%	11 30%	24 70%	30 71%	12 29%
IV........	11 85%	2 15%	8 66%	4 34%	8 61%	5 39%	6 75%	2 25%	10 80%	3 20%
All types of tubercular patients....	63 61%	39 39%	62 70%	27 30%	51 52%	48 48%	33 39%	52 61%	66 66%	33 34%

In the first series, 98 per cent positive results in the type III cases was reported. The results in the five recent series of type III cases show 84 per cent in the second series, 92 per cent in the third series, 87 per cent in the fourth series, 84 per cent in the fifth series and 90 per cent in the sixth series. The difference in the percentage of positive results which have been obtained in this type of tuberculosis patients is probably due to the differences in the classifications made by the various diagnosticians.

The results of all tests in these six series tabulated on Table 15 demonstrate the fact that the moderately and far advanced cases in good condition showing constitutional symptoms (type III) gave uniformly the highest percentage of positive reactions, and that both the incipient cases with constitutional symptoms (type II) and the advanced cases with very poor prognoses (type IV) gave irregular results. The patients exhibiting few if any symptoms regardless of the stage of the disease (type I) gave uniformly the lowest precentage of positive reactions. The clinically non-tuberculous cases gave only 1.7 per cent positive fixations.

TABLE 20B

Report of all fixations test on the Otisville cases (fifth series) using the No. 170 antigen

Number of type I, II, III and IV cases giving + reactions... 88 or 73 per cent

Number of type I, II, III and IV cases giving ± or − reactions... 33 or 27 per cent

TYPES	RESULTS	
	+	− or ±
Sera from non-tuberculous controls..................	2 3%	68 97%
Suspects (doubtful cases)...........................	1 25%	3 75%
I..	25 60%	17 40%
II...	7 60%	5 40%
III..	43 84%	8 16%
IV...	13 80%	3 20%

TABLE 21

Report of the sixth series

Number of complement fixation tests made	1516
Number of sera examined	230
Number of patients examined	201
Number of clinically tuberculous patients	61
Number of clinically non-tuberculous patients	140
Number of Type 1, 2, 3 and 4 cases giving + reactions	41 or 73.2 per cent
Number of Type 1, 2, 3 and 4 cases giving ± or − reactions	15 or 26.8 per cent

NUMBER	TYPE	POSITIVE		DOUBTFUL AND NEGATIVE	
		Number	Per cent	Number	Per cent
154	Non-tuberculous	2	1.4	152	98.6
6	Suspects	2	33.0	4	66.0
14	Inactives	3	21.0	11	79.0
9	I	2	23.0	7	77.0
11	II	8	73.0	3	27.0
31	III	28	90.0	3	10.0
5	IV	3	60.0	2	40.0

15. THE VALUE OF THE COMPLEMENT FIXATION REACTION TO THE CLINICIAN IN THE DIAGNOSIS AND PROGNOSIS OF TUBERCULOSIS

When all is said and done, the final answer to the question of the value of this reaction to the clinician will be given by the clinicians themselves in either using or discarding this aid to diagnosis.

The tuberculosis specialist will probably say, "We can make our own diagnoses with the aid of auscultation and the signs and symptoms of the patient in practically all active tuberculosis cases except the very early ones with lesions too small to detect and with practically no symptoms." They will then ask, "Will this test give positive reactions in a large percentage of our very early cases with practically no symptoms?" The answer is, "No." In such cases the fixation reaction will be positive in only a small percentage, possibly from 25 to 40 per cent. The specialist will also ask, "Can you be sure of your prognoses in all cases by reading this test?" Again the answer will be, "No." It will be of value only as a prognostic aid when it is added to the clinical data already available. The above two answers will cause many of the specialists to reject the test as of no value to them except as confirmatory evidence.

However, this fixation tests will be of the utmost value to the general practitioner who very frequently calls a case incipient, when the specialist would classify it as moderately advanced. Many cases, with small lesions but with moderate symptoms—hemorrhage, night sweats, afternoon temperature, etc., come to the practitioner. No tubercle bacilli can be demonstrated in the sputum and he thinks, but is not at all certain, that his case has tuberculosis. This is the type of case that the complement fixation test will pick up and by repeated positive findings prove the presence of an active tuberculous focus.

Again, as an aid in prognosis this test will be of marked value to the average clinician if he uses it intelligently along with his clinical findings; for instance, if he follows with the fixation test, at rather frequent intervals, a moderately advanced case in apparently good clinical condition, a weakening of the reaction from a strong positive to a weak positive and finally a negative reaction is a very good prognostic sign for the time being. Then as everyone knows some outside factor may later complicate the case and light up the healed lesion into fresh activity. The complement fixation reaction under such a condition would at once become positive again.

Or if he follows a far advanced case in a very poor clinical condition; the sudden weakening of the reaction from a strong positive to a weak positive or negative reaction would be a very poor prognostic sign, indicating the loss of resistance; i.e., the power of the body to elaborate antibodies is lost, or these antibodies are overwhelmed by the excess of antigen thrown into the blood stream.

The clinician will ask to what degree in each class of cases does the complement fixation reaction help in diagnosis and treatment beyond clinical and sputum examinations? To this the reply must be—it will be of very great diagnostic aid to the average practitioner in the incipient cases showing symptoms but with no or only few definite signs and with negative sputum reports. The reaction in such cases will be positive in probably 70 to 90 per cent.

In the moderately and far advanced cases in good condition showing symptoms, it will be only of diagnostic value, of course, as a confirmatory test, being positive in from 80 to 90 per cent. In these cases, if made frequently and used with the clinical data, it will be of great aid in prognosis.

In the advanced cases in poor condition it will give very irregular results and is of most value as a prognostic aid.

In the cases having few or no symptoms, no matter what stage the disease is in, the reaction is practically worthless, as the percentage of positive results is only from 25 to 50 per cent. In the general summing up of its appearance in all types of cases (active) one finds that it is comparable with the Wassermann reaction in syphilis.

Gradwohl (101) states that he, like others, has been forced to conclude that a positive Wassermann test is laboratory proof of syphilis, but that a negative Wassermann is by no means a method of proving that syphilis does not exist. He concludes that of all treated syphilitics examined, only 60 per cent have given him positive findings.

Snow and Cooper (102) conclude that the sera of non-syphilitic tuberculous patients may give partial to complete Wassermann reactions when cholesterinized antigens are used in about 31 per cent of the cases.

Lewis and Newcomer (103) state that the Wassermann reaction is positive in other conditions, but that it is not so generally recognized. They cite fresh instances of this in certain febrile cases. They also conclude that a positive Wassermann reaction fails to appear in a considerable percentage of syphilitics.

What the future will bring is hard to say. In all probability no better results can be expected from the complement fixation test based on the specific tuberculous antibodies in the patient's blood serum than have already been reported by numerous serologists.

TABLE 22

Clinical and laboratory report of the Otisville series

NUMBER	NAME	SPUTUM REPORT[4]	FIRST OTISVILLE CLASS[1]	SECOND OTISVILLE CLASS[2]	WRITER'S CLASSIFICATION[3]	REPORTS WITH NO. 170 ANTIGEN		REPORTS WITH NO. 319 ANTIGEN		REPORTS WITH NO. 125 ANTIGEN
						First specimen	Second specimen	First specimen	Second specimen	
1	C. Nicolelle	+[8]	1A	[6]	1	−		−		+
2	E. Klein	0[9]	0	1A	1	−		−		±
3	Bessie Horowitz	±[10]	2B	2B	3	−	2+	−	−	−
4	D. Smith	−[11]	1A		Susp.	−		−	−	−
5	V. Kreis	−	2A		1	−		−		−
6	C. Zikarsky	−	1A		Susp.	±		±		2+
7	R. Burns	−	1A	1A	1	−	±	2+	+	±
8	H. Shapiro	−	1A		Susp.	+		±		+
9	M. Rosch	−	1A	1A	1	−	−	−	−	−
10	M. Berman	−	2A	2A	1	−	±	+	−	±
11	L. Gebbardt	+	2A	2B	3	3+	4+	2+	+	3+
12	C. Sullivan	+	2A	2A	1	−	+	−	±	+
13	C. Kircher	−	2A		1	−		−		±
14	R. Anerheim	−	1A		Susp.	−		−		±
15	Mary Cleary	+	3A	3A	1	4+	3+	4+	3+	3+
16	C. O'Donnell	+	2A	2B	3	2+	4+	0	3+	3+
17	E. Wagenbach	+	2B		3	±		−		±
18	A. Rifkin	−	2A		1	±		−		±
19	M. Kenny	−	2A	1A	1	+	−	0	±	±
20	R. Gachino	+	2B		3	2+		±		2+
21	N. Donohue	+	3A	2A	1	4+	4+	3+	3+	4+
22	A. Drennan	−	1A		1	−		−		+
23	A. Kiley	−	1A	1A	1	±	−	−	−	4+
24	J. Frey	0	2B		3	4+		4+		4+
25	A. Wood	+	2A	2A	1	4+	4+	4+	4+	4+
26	J. Healy	+	1A	2C	4	2+	+	−	−	2+
27	M. Bachman	+	3B	3C	4	4+	4+	4+	4+	4+
28	M. Kroin	+	2B		3	2+		0		0
29	H. Ferrera	+	3B		3	4+		4+		4+
30	M. Mulhalley	−	1A		1	±	+	−	−	±
31	M. Clyner	+	3A		1	4+		4+		4+
32	B. Wolff	+	2A		1	3+		4+		4+
33	C. McGovern	+	2C	2C	4	4+	4+	+	4+	4+
34	A. Constantine	+	2B		3	+		±		4+
35	A. Ficcarrota	+	2B	2C	4	4+	4+	+	4+	4+
36	J. Kreiger	−	1A	1A	1	−	−	−	−	−
37	J. Rossling	+	1A	2B	3	4+	4+	4+	4+	4+
38	O. Ogren	+	2A	2C	4	2+	−	0	−	+

TABLE 22—*Continued*

NUMBER	NAME	SPUTUM REPORT[4]	FIRST OTISVILLE CLASS[1]	SECOND OTISVILLE CLASS[2]	WRITER'S CLASSIFICATION[3]	REPORTS WITH NO. 170 ANTIGEN		REPORTS WITH NO. 319 ANTIGEN		REPORTS WITH NO. 125 ANTIGEN
						First specimen	Second specimen	First specimen	Second specimen	
39	J. Kaplan	–	3B	3B	3	–	–	–	–	+
40	A. Higgins	+	4C		3	2+	4+	3+	3+	4+
41	E. Roder	+	3B		3	4+		4+		4+
42	R. Antonio	+	2A	2A	1	+	+	±	–	3+
43	F. Maklary	+	3C	3C	4	4+	3+	+	2+	4+
44	J. Becker	+	1B	1B	2	±	–	–	–	–
45	J. T. Searoni	+	2B		1	2+	±	4+	+	2+
46	H. Hukenson	+	1B	1B	2	4+	4+	4+	4+	4+
47	J. Brown	+	3A	3A	1	4+	4+	4+	4+	4+
48	V. Olsen	+	1B	1B	2	2+	3+	2+	–	2+
49	W. Blay	+	2B	2B	3	+	+	±	–	3+
50	G. DeShay	+	2C	2C	4	4+	4+	4+	4+	4+
51	J. Malcolms	–	2B	2B	3	+	3+	+	+	±
52	A. Miller	–	2A	2A	1	3+	4+	2+	3+	4+
53	J. Coffin	–	2B	2B	3	4+	4+	4+	2+	4+
54	S. Hickey	+	2C	2A	1	+	3+	2+	–	3+
55	W. Trabuci	+	1A		1	4+	3+	4+	3+	4+
56	H. Hageman	+	2B	2B	3	2+	3+	2+	–	+
57	H. Kildea	+	3C	3C	4	–	–	–	–	ac
58	J. Schneider	–	2B	2B	3	–	+	±	–	–
59	L. McGann	–	2B	2B	3	–	±	±	–	+
60	J. Dunn	+	2B	2B	3	–	+	–	±	0
61	J. Atty	+	2B	2B	3	4+	4+	2+	2+	2+
62	A. Glassheim	+	1A	1A	1	4+	2+	4+	+	4+
63	A. Larghi	+	2B	2B	3	4+	3+	–	±	4+
64	L. Weisberg	+	1A	2A	1	±	–	–	–	±
65	E. Anderson	+	2B	2B	3	–	+	–	–	–
66	H. Swiss	+	3B		3	+		–		–
67	A. Steislet	+	1A		1	3+		4+		4+
68	A. Torak	+	4C		4	4+		3+		3+
69	C. Seedyk	+	2A	2A	1	+ac	±	–	–	0
70	J. Alletsee	+	2B	2A	1	2+	3+	±	–	3+
71	F. Frascino	–	2B	2B	3	±	+	+	–	2+
72	H. Slusek	+	1B	1B	2	+	+	–	2+	±
73	S. Naroty	–	1B	1B	2	2+	2+	–	–	±
74	G. Cleaver	–	2B	2B	3	2+	3+	3+	±	±
75	H. Franklin	–	2B	2A	1	–	+	–	–	–
76	S. Scott	–	2B	2A	1	±	–	–	–	±

TABLE 22—*Continued*

NUMBER	NAME	SPUTUM REPORT[4]	FIRST OTISVILLE CLASS[1]	SECOND OTISVILLE CLASS[2]	WRITER'S CLASSIFICATION[3]	REPORTS WITH NO. 170 ANTIGEN		REPORTS WITH NO. 319 ANTIGEN		REPORTS WITH NO. 125 ANTIGEN
						First specimen	Second specimen	First specimen	Second specimen	
77	H. Lavelle	+	2B	2B	3	±	2+	±	–	3+
78	J. Willis	+	2B	2B	3	±	2+	–	–	4+
79	G. Gould	–	3B		3	4+		4+		4+
80	R. McDonnell	+	2B	2B	3	+	4+	±	±	4+
81	N. Kelly	+	1C	1C	2	4+	3+	±	±	4+
82	W. Day	–	1B	1B	2	±	±	–	–	+
83	J. Pestrok	+	4C		4	+		–	–	±
84	J. Patten	+	2C	2B	3	–	–	–	–	–
85	J. Como	+	2B	2B	3	+	±	–	±	+
86	M. Chipront	+	2C		2	+		±	–	+
87	H. Robinson	–	1B	1B	2	–	–	–	–	–
88	N. Cheturachino	–	2B	2B	3	+	2+	+	±	±
89	J. Kaminski	+	2B	2B	3	4+		4+		4+
90	E. Murmane	+	3B	2B	3	–	–	±	–	4+
91	W. Kirk	–	1B	1B	2	–	–	–	±	±
92	B. J. Walsh	+	2C	2B	3	+	2+	+	–	2+
93	H. Pearson	+	2B	2A	1	±	+	±	±	4+
94	A. Bauer	+	2B	2B	3	+	+	3+	+	3+
95	J. Walsh	+	3B		3	–		–		±
96	G. Murganeo	–	2B		2	±		+		3+
97	H. Darci	–	2C	2C	4	–	–	–	–	–
98	W. Hunt	+	4C		4	4+		2+		4+
99	J. Christ	+	3B		3	2+		+		±
100	W. Allen	+	3B		3	4+		4+		4+
101	P. Masterson	+	3B	3B	3	–	±	–	–	–
102	F. Bittanto	+	2B	2B	3	2+	+	2+	±	2+
103	J. Devlin	+	4C		4	3+		4+		2+
104	J. McDonald	–	NT[12]	NT	NT	–	–	–	–	–
105	Geo. Early	–	NT	NT	NT	–	–	–	–	–
106	F. Cahill	–	NT	NT	NT	2+	±	–	–	2+
107	Ed. Sweeny	–	NT	NT	NT	–	–	–	–	–
108	J. Reilly	–	NT	NT	NT	–	–	–	–	–
109	J. Outney	–	NT	NT	NT	–	–	–	–	–
110	G. Bowers	–	NT	NT	NT	–	±	±	±	±
111	W. Liber	–	NT	NT	NT	±	2+	–	2+	4+
112	Cleary	–	NT	NT	NT	–	–	–	–	±
113	Wm. Pienery	–	NT	NT	NT	–	–	–	–	–
114	J. Mahony	–	NT	NT	NT	–	–	–	–	–

TABLE 22—*Concluded*

NUMBER	NAME	SPUTUM REPORT[7]	FIRST OTISVILLE CLASS[1]	SECOND OTISVILLE CLASS[2]	WRITER'S CLASSIFICATION[3]	REPORTS WITH NO. 170 ANTIGEN		REPORTS WITH NO. 319 ANTIGEN		REPORTS WITH NO. 125 ANTIGEN
						First specimen	Second specimen	First specimen	Second specimen	
115	Wm. Lent	+			TBA[13]	4+	4+	3+	2+	4+
116	C. Gaynor	−	NT	NT	NT	−	−	−	−	−
117	P. Mills	−	NT	NT	NT	−	2+	−	−	4+
118	McDermott	−	NT	NT	NT	−	−	−	−	−
119	Foster	−	NT	NT	NT	−	±	−	±	−±
120	Angelo	−	NT	NT	NT	−	−	−	−	−
121	De Laney	−	NT	NT	NT	−	−	−	−	−
122	McNold	−	NT	NT	NT	−	−	−	−	−
123	J. Colbert	−	NT	NT	NT	±	−	−	−	±
124	Lottie Crews	−	NT	NT	NT	−	−	−	−	−
125	Hansen	−	NT	NT	NT	−	−	−	−	−

[1] Refers to the classification made by the clinician at the time the first specimens were obtained.

[2] Refers to the subsequent classification made by the clinician three weeks later when the second specimens were taken.

[3] Refers to the writer's classification of the cases. This was obtained by transcribing the first Otisville (National classification) into terms of the writer's classification.

[4] Refers to the first specimens taken from the patients.

[5] Refers to the second specimens taken three weeks later from the same patients.

[6] Blank space indicates that only one specimen of serum was obtained from the patient.

[7] Sputum report obtained within one month of the time the blood specimen was taken.

[8] Refers to the finding of many or moderate numbers of tubercle bacilli.

[9] No specimen examined for quite a long while.

[10] Refers to the finding of only an occasional bacilli.

[11] Remaining non-tubercular control sera were negative in all tests with all antigens.

[12] NT refers to non-tuberculous.

[13] TBA,—Tuberculous arthritis of the ankle.

Explanation of the above table: The foregoing table gives a résumé of the important points noted in the Otisville investigation (series V). In addition to the foregoing data, we had von Pirquet tests, and Wassermann reactions on quite a few and clinical data on all.

16. SUMMARY

The one hour fixation period in the water bath at 37°C. appears to be the optimum time and temperature for the complement fixation test with our antigens.

Specially tested complement, if only one guinea-pig is killed should be used, but untested pooled complement from six or more guinea-pigs apparently gives satisfactory results.

Natural antisheep hemolysin is markedly thermolabile.

If the serum is kept sterile the natural antisheep hemolysin does not depreciate very rapidly due to ageing.

Heating tubercle bacillus antigen at 100°C. for three hours does not seem to impair its antigenic value in the slightest. The addition of 25 per cent alcohol or ½ per cent carbolic acid to the finished antigen as preservative tends to make the antigen anticomplementary to a greater or less degree.

The best method for preparing the tubercle bacillus antigen seems to be by killing the bacilli with alcohol, and after the finished antigen is bottled and corked in small vials, sterilizing at 100°C. for one hour on two or three successive days to kill all contaminating spores and bacteria.

An increase in the percentage of positive results can probably be obtained by absorption of the natural antisheep amboceptor before making the test or by using the fresh non-inactivated sera according to the technic advocated by Hecht and Gradwohl. However, while both of these modifications give a higher percentage of specific positive reactions than the standard test, they also give a moderate percentage of non-specific reactions. Therefore, they should only be used as control tests along with the standard technic.

The increase in the strength of the fixation and the increase in the percentage of positive findings after preserving the patient's sera for seven days in the ice chest is due, probably, to one or more of the following reasons: the formation of thermostable antilysins in the kept sera, loss of natural antisheep amboceptor due to ageing, loss of natural antisheep amboceptor due to reheating and other unknown non-specific causes. This change in sera after preserving in the ice chest for one week is apparently non-specific.

No cross fixation was apparent between syphilitic antibodies and the tubercle bacillus antigens which were used in this study.

The results of the von Pirquet reaction apparently do not parallel the results obtained with the complement fixation test.

The National Association Classification of tuberculosis patients appears to be too complicated to be used in successfully interpreting this test. If used in its simplified form, the results of the test do not run parallel with the classification.

By using the writer's simplified interchangeable classification a more accurate interpretation of the results of this test is possible. However, even with this simpler classification it was found impracticable accurately to tabulate the results by types in a routine way as the classification of these same patients varied considerably when made by different clinicians. In general, we may say that the largest percentage of negative and weak positive results are in the cases having few or no constitutional symptoms, while the strength and percentage of positive reactions increase progressively with the increased constitutional symptoms, until the patient reaches the far advanced condition, when the reverse is true; i.e., when the patient loses his power of resistance, the complement fixation reaction becomes negative.

The age of the patient apparently has no effect on the complement fixation test for tuberculosis.

About 70 per cent of all types of tuberculosis patients except those clinically healed or inactive gave positive fixation results on repeated tests. Normal non-tuberculous cases gave almost no positive results on repeated tests. Moderately and far advanced cases in good condition showing constitutional symptoms (type III) gave an average of 85.2 per cent positive fixations for all six series.

This complement fixation reaction will not be very valuable, as an aid in diagnosis, to the tuberculosis specialist except as a confirmatory test. However, a positive fixation reaction will be of very great value to the general practitioner not only as a confirmatory test but also as an aid in diagnosis and prognosis.

17. CONCLUSIONS

The results of 6128 complement fixation tests made on 1207 sera from 1000 patients point to the fact that this is not a 100 per cent test for the diagnosis of tuberculosis. A considerable percentage of sera from incipient and far advanced cases apparently contain insufficient antibodies to fix complement, no matter what system or what antigen is used for the test. This fact, therefore, precludes the probability of a 100 per cent test based on complement binding antibodies in the patient's serum.

A great many antigens and many systems have been tried by various workers but even the most favorable reports on large series bear out this statement.

Numerous favorable reports have been published on small series but apparently none have been confirmed where the percentage of positive findings, with a series of unselected active tuberculous cases of all types, has been more than 80 per cent—unless there was at the same time a marked degree of non-specific fixation.

About 70 per cent positive results appears to be the average findings, with all types of unselected active tuberculous cases, for many thousands of complement fixation tests made by many serologists, using tubercle bacillus suspensions or tuberculins as antigens. The reactions are weakest when the patient exhibits few, if any, symptoms of tuberculosis, while they are most definite and strongest in the incipient and moderately advanced cases exhibiting marked symptoms. The results are therefore more confirmatory than actually diagnostic in the largest percentage of cases. However, when used intelligently along with the clinical history, the results justify its more extended use.

A positive reaction repeated twice seems to prove fairly conclusively that the patient has an active tuberculous process. The weakening of the reaction from a strong positive to a weak positive or negative reaction, is apparently a good prognostic sign in incipient and moderately advanced cases exhibiting clinical improvement, while at the same time this same change with a far advanced case in a poor clinical condition is a very bad prognostic sign.

REFERENCES

(1) McCrae and Funk: Jour. Am. Med. Assoc., 1919, **73**, 161.
(2) Widal and LeSourd: Cited by Shennan and Miller. Edinburgh Med., Jour., 1913, **10**, 81.
(3) Bordet and Gengou: Compt. rend. Acad. de Sc., 1903, **137**, 351.
(4) Wassermann and Bruck: Deutsch. med. Wchnschr., 1906, **32**, 449.
(5) Caulfield: Arch. Int. Med., 1911, **8**, 440.
(6) Caulfield and Beattie: Jour. Med. Research, 1911, **24**, 122.
(7) Deilmann: Zeit. f. Immunitätsf., 1911, **10**, 421.
(8) Laird: Jour. Med. Research, 1912, **27**, 163.
(9) Möllers: Cent. Bact., Ref., 1912, **54**, Beiheft, 202.
(10) Hammer: Deutsch. Tierarzt. Woch., 1912, **20**, 593.
(11) Zweig: Berl. klin. Woch., 1912, **49**, 1845.
(12) Calmette, Massol and Mézie: Compt. rend. Soc. Biol., 1912, **73**, 122
(13) Much: Munch. med. Wchnschr., 1912, **59**, 685.
(14) Letulle: Thèse, Fac. Méd. Paris, 1912.
(15) Fraser: Ztschr. f. Immunitätsf., Orig., 1913, **20**, 291.
(16) Dudgeon, Meek and Weir: Lancet, 1913, **1**, 19.
(17) Bang and Andersen: Cent. Bact., Orig., 1913, **69**, 517.
(18) Armand-Delille, Rist and Vaucher: Compt. rend. Soc. Biol., 1913, **74**, 791.
(19) Wyschelessky: Zeit. f. Tuberk., 1912, **19**, 209.
(20) Kinghorn and Twitchell: Zeit. f. Tuberk., 1913, **20**, 11.
(21) Rothe and Bierbaum: Deutsch. Med. Woch., 1913, **39**, 644.
(22) Harris and Lanford: Jour. Inf. Dis., 1913, **13**, 301.
(23) Momose: Deutsch. Med. Woch., 1913, **39**, 1029.
(24) Besredka: Zeit. f. Immunitätsf., Orig., 1914, **21**, 77.
(25) Wwedensky (revised by Wulffius): Zeit. f. Immunitätsf. referat., 1914, **8**, 931.
(26) Debains and Jupille: Compt. rend. Soc. Biol., 1914, **76**, 199.
(27) Kuss, Leredde and Rubinstein: Ibid., 244.
(28) Inman: Compt. rend. Soc. Biol., 1914, **76**, 251.
(29) Bierbaum and Berdel: Zeit. f. Immunitätsf., 1914, Orig., **21**, 249.
(30) McIntosh and Fildes: Lancet, 1914, **2**, 485.
(31) Radcliffe: Lancet, 1914, **2**, 488.
(32) Dudgeon, Meek and Weir: Jour. Hyg., 1914, **14**, 52, 72.
(33) Dudgeon, Meek and Weir: Jour. Hyg., 1914, **14**, 52, 72.
(34) Bronfenbrenner: Arch. Int. Med., 1914, **14**, 786.
(35) Bronfenbrenner: Proc. Soc. Exp. Biol. and Med., 1914, **11**, 180.
(36) Bronfenbrenner: Proc. Soc. Exp. Biol. and Med., 1914, **11**, 92–93.
(37) Bronfenbrenner and Schlesinger: Trans. 12th Meet. Nat. Assoc. Study and Prev. Tuberc. 1916, 225.
(38) Craig: Am. Jour. Med. Sc., 1915, **150**, 781.
(39) Stimson: Bull. Hyg. Lab. U. S. P. H. S., 1915, **101**, 7.
(40) Corper: Jour. Inf. Diseases, 1916, **19**, 315.
(41) Miller, H. R., and Zinsser, H.: Proc. Soc. Exper. Biol. and Med., 1916. **13**, 134.

(42) Miller, H. R.: Jour. Am. Med. Assoc., 1916, **67**, 1519.
(43) Woods, Bushnell, and Maddux: Jour. of Immunol., 1917, **2**, 301.
(44) McCaskey, G. W.: Am. Jour. Med. Sc., 1917, **154**, 648.
(45) Brown and Petroff: Am. Rev. Tuberc., 1918, **2**, 525.
(46) Lange, L. B.: Am. Rev. Tuberc., 1918, **2**, 541.
(47) Stivelman, B.: Amer. Rev. Tuberc., 1918, **2**, 546.
(48) Lewis, P. A.: Am. Rev. Tuberc., 1919, **3**, 129.
(49) Pritchard and Roderick: Jour. Am. Med. Assc., 1919, **73**, 1879.
(50) Cooke, J. V.: Jour. of Inf. Dis., 1919, **25**, 493.
(51) Stoll and Neuman: Jour. Am. Med. Assc., 1919, **72**, 1043.
(52) Moursund, W. H.: Jour. Inf. Dis., 1920, **26**, 85.
(53) Moon, V. H.: Jour. Am. Med. Assc., 1919, **71**, 1127.
(54) Bronfenbrenner: Jour. Lab. Clin. Med., 1917, **3**, 50.
(55) Boez and Duhot: Compt. Rend. Soc., Biol., 1919, **82**, 559.
(56) Wilson, M. A.: Jour. Immunol., 1918, **3**, 345.
(57) Ruediger, E. H.: Jour. Inf. Diseases, 1919, **24**, 405.
(58) Noguchi, H., and Bronfenbrenner: Jour. Exper. Med., 1911, **13**, 217.
(59) Ronchese, A. D.: Compt. Rend. Soc. Biol., 1919, **82**, 193.
(60) Thompson: Jour. Am. Med. Assc., 1916, **66**, 652.
(61) Rhamy: Jour. Am. Med. Assc., 1917, **69**, 973.
(62) McNeill (cited by Thompson): Amer. Jour. of Syph., 1917, **1**, 555.
(63) Kolmer: Jour. of Immunol. to be published.
(64) Thiele, F. H., and Embleton, D.: Ztsch. f. Immunitätsf. U. Exper. Ther., Orig., 1914, **20**, 1–50.
(65) Sherman, H.: Jour. Inf. Dis., 1918, **22**, 534.
(66) Miller, H. R.: Jour. of Lab. and Clin. Med., 1916, **1**, 816.
(67) Corper: Jour. Inf. Diseases, 1916, **19**, 315.
(68) Brown and Petroff: Amer. Rev. Tuberc., 1918, **2**, 525.
(69) Bronfenbrenner, Kahn, M. H., Rockman and Kahn, M.: Arch. Int. Med., 1916, **17**, 492.
(70) Bruce, W. J.: Jour. Lab. and Clin. Med., 1919, **4**, 215.
(71) Lewis and Newcomer: Jour. Exp. Med., 1919, **29**, 351.
(72) Gradwohl, R. B. H.: Jour. Am. Med. Assc., 1917, **68**, 514.
(73) Rubinstein: Compt. Rend. Soc. Biol., 1919, **82**, 526.
(74) Rubinstein and Radossavlievitch: Compt. Rend. Soc. Biol., 1919, **83**, 361.
(75) Eschbach and Duhot: Compt. Rend. Soc. Biol., 1919, **82**, 452.
(76) Bauer, J.: Deutsch. Med. Wchnschr., 1908, **34**, 698.
(77) Bauer, J.: Berl. klin. Wchnschr., 1908, **45**, 834.
(78) Rossi, O.: Ztsch. f. Immunitätsf., orig., 1911, **10**, 321.
(79) Simon, C. E.: Jour. Am. Med. Assoc., 1917, **72**, 1535.
(80) Wechselmann: Ztsch. f. Immunitätsf., orig., 1909, **3**, 525.
(81) Noguchi, H., and Bronfenbrenner: Jour. Expr. Med., 1911, **13**, 217.
(82) Jacobaeus, H. C.: Ztsch. f. Immunitätsf., orig., 1911, **8**, 615.
(83) Olmstead, M. P.: Medical Record, N. Y., 1914, **85**, 341.
(84) Van Saun, A. I.: Jour. Lab. and Clin. Med., 1917, **3**, 59.
(85) Ottenberg, R.: Archiv. Int. Med., 1917, **19**, 457.
(86) Seelman, J. J.: Jour. Lab. and Clin. Med., 1918, **3**, 626.

(87) STERN, M.: Ztsch. f. Immunitätsf., orig., 1914, **22**, 130.
(88) STIMSON: Bull. Hyg. Lab. U. S. P. H. S., 1915, **101**, 7.
(89) NEILL, M. H.: Hygienic Laboratory Bulletin No. 110, September 1917, 47.
(90) RAPOPORT, F. H.: Jour. Am. Med., Assc., 1919, **72**, 633.
(91) BURNS, SLACK, CASTLEMAN AND BAILEY: Jour. Am. Med. Assc., 1917, **68**, 1386.
(92) BRONFENBRENNER: Jour. Exp. Med., 1912, **15**, 598.
(93) CORPER AND SWEANY: Jour. Am. Med. Assoc., 1917, **68**, 1598.
(94) BOEZ AND DUHOT: Compt. Rend. Soc., Biol., 1919, **82**, 559.
(95) TURBAN: Diagnosis of Tuberculosis of the Lung, Edition 1906, 42–49.
(96) TURBAN: Reference quoted by Rathbun; not found.
(97) MEISSEN: Handbuch der Tuberculose, Band 1,743, published by Barth, Leipzig, 1914.
(98) RATHBUN, W. L.: N. Y. C., Dept. of Health. Tuberculosis Monograph, No. 4, March ,1917.
(99) VON WEDEL, H.: Jour. Immunol., 1918, **3**, 351.
(100) MOURSUND, W. H.: Jour. Inf. Dis., 1920, **26**, 85.
(101) GRADWOHL, R. B. H.: Jour. Am. Med. Assc., 1914, **63**, 240.
(102) SNOW AND COOPER: Amer. Jour. of Med. Sc., 1916, **152**, 185.
(103) LEWIS AND NEWCOMER: Jour. Exp. Med., 1919, **29**, 351.
(104) HAMMER: Munch. Med. Wchnschr., 1912, **59**, 1750.
(105) DUDGEON, MEEK AND WEIR: Jour. Hyg., 1914, **14**, 52, 72.
(106) BESREDKA: Compt. rend. Acad. d. sc., 1913, **156**, 1633.
(107) CALMETTE AND MASSOL: Compt. rend. Soc. Biol., 1913, **75**, 160.
(108) LUCKE, B.: Jour. Immunol., 1916, **1**, 457.
(109) PORTER: Jour. Hygiene, 1911, **11**, 105.

ON THE TRANSFER OF THE SO-CALLED NORMAL-ANTIBODIES FROM MOTHER TO OFFSPRING

I. AGGLUTININS

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By far the greater number of those who have occupied themselves with experiments on the agglutinins (bacterial as well as hemagglutinins) present in the blood of normal mothers and their newborn young, have arrived at the result that in the blood of the offspring these bodies are absent or at any rate less in amount than in that of the mother, despite the fact that the mother's serum as well as the mother's milk shows a strong reaction. This conclusion was reached by Gruenbaum (1), Halban (2), Halban and Landsteiner (3), Schumacher (4), Schenk (5), Zubrezychi and Wolfsgruber (6), for human beings, and by Kraus and Loew (7), Kraus and Clairmont (8), G. Mueller (9), Park (10), Luedke (11), V. Eisler and Sohma (12) for various species of animals, whereas Klieneberger (13) draws no quantitative conclusions; finally V. Fellenberg and Doell (14) arrive at the result, that the serum of a child sometimes shows a stronger, sometimes a weaker reaction than that of the mother, and they decline to believe in any constant ratio between them.

According to Pfaundler (15), the fact that the serum of the new-born agglutinates faintly or not at all, necessitates, in view of the Gruber-Widal reaction, the fixation of the upper limit of the normal agglutination for the various ages.

The mother's milk was found by Schumacher to contain agglutinin in varying amounts; Fellenberg and Doell tested the proportion between the agglutinin content of the mother's serum and milk, but could not demonstrate any constant proportion, and Zubrezychi and Wolfsgruber found in human milk rather large amounts of hemagglutinin, which, in their opinion, is not transmitted to the serum of suckling children. Whereas the relative agglutinin content of the blood of mother and offspring at the moment of birth is, in the main, clearly elucidated through the works quoted above, the time immediately after the birth of the young has, to all intents and purposes, been treated only in the above mentioned, by no means exhaustive, work by Zubrezychi and Wolfsgruber. The reason of this is to be sought in the fact that the researches are chiefly based upon human material or have been undertaken with small experimental animals; hence, a regular taking of blood samples at short intervals has been in both cases out of the question.

The object of this work was, through regular series of blood and milk tests, to examine the quantitative proportion of the agglutinins (normally present in the blood) in the blood and milk of the mother and the blood of the offspring, in order to fix the relative values of these three. As the experimental animal, the goat was selected, it being in *every* respect suitable for the purpose; the samples of blood were taken from the jugular vein.

The samples were tested for typhoid and coli agglutinin as well as for agglutinin against horse and rabbit blood-corpuscles; each blood sample was tested on as many as possible of these four. As the measurements of bacterial agglutinin is impossible in the natural milk, it is necessary to make this fluid translucent by precipitation of the casein by means of rennet; all the figures below are given for this milk serum.

The technic followed in these experiments was the one used at the institute and originally introduced by Th. Madsen and Jorgensen (16) for the determination of bacterial agglutinins, and the same method was employed for the measuring of the hemagglutinins, only with somewhat different quantitative proportions; thus 8 cc. of 1 per cent blood suspension per tube were used in the tests for hemagglutinin, and only 1 cc. of bacterial suspension was used in the bacterial tests.

In view of the tests undertaken at the same time for normal antihemolysins, which will be communicated in a later article, the samples were inactivated for half an hour at 56° with the exception of kid 7 and 8 (see table 1), after experiments had been carried out showing that the weakening was imperceptible at this temperature. Only at higher temperatures does the influence of heat become perceptible, as was formerly shown by Th. Madsen and Streng (17) in the case of the immune agglutinins.

All of the kids were weighed at the taking of the blood samples, but the increase in weight has not been taken into consideration in the calculation, because the period within which their blood contains agglutinin is so short.

Nor was the hydrogen ion concentration taken into consideration during the measuring of the agglutinin, for, as demonstrated by Michaelis and Davidsohn (18), fluctuations in pH round the neutral point plays no part, and I have made use of a broth the pH of which lay at this point, and which was, besides, very rich in regulators; furthermore, in the case of the hemagglutinins there have always been rather large quantities of serum in each tube and according to Walbum's (19) researches serum is an excellent regulator when present in sufficient concentration.

In table 1 is presented a summary of the researches which were made on 7 pairs of twins. Dates of the bleeding and of the milking are given. The results are quoted in agglutinin units (content per cubic centimeter); the dates of the birth are marked with an asterisk. It appears from table 1 that only the serum of one kid contained agglutinin at birth, in spite of the fact that in all of the cases agglutinin was present in the serum of the mother animal. This case may possibly be a parallel to the few instances in which previous experimentators, Gruenbaum and Schumacher find "inherited" agglutinin. The agglutinin is transmitted to the kid through the milk, as appears with probability from the table; in the milk as well as in the kid's serum it decreases quickly, and as a rule it first disappears from the milk. The titer in the serum of the kid may be above or below that in the mother's serum, but only in a single case, where the milk gave an abnormal reaction (12 and 13's mother), was the titer higher than that of the milk, which was possibly due to the presence of bodies preventing agglutination in the milk, such as those previously

TABLE 1

KID NUMBER	TEST MADE FOR	MOTHER'S SERUM																		
7 and 8	Typhoid agglutinin.	25/9	22/11	6/2	12/2	21/2*	23/2	25/2	28/2	3/3	7/3	11/3	16/3	20/3						
		5.4	3.6	4.4	5.4	4.4	5.0	5.0	4.4	3.6	3.6	3.6	2.5	3.3						
9 and 10	Typhoid agglutinin.	22/9	22/11	6.2	12/2	24/2*	26/2	28/2	3/3	7/3	11/3	14/3	17/3	21/3	25/3	30/3				
		2.5	2.2	2.0	2.3	2.4	2.5	2.8	2.5	2.5	2.2	2.2	2.2	2.2	2.5	2.8				
12 and 13	Horse blood agglutinin.	25/9	12/12	12/2	25/2	27/2*	3/3	5/3	7/3	11/3	17/3	21/3	25/3	28/3	2/4	7/4				
		3.3	5.4	4.5	3.3	2.5	3.3	2.7	5.4	2.5	2.5	2.5	2.7	2.7	3.3	2.7				
18 and 19	Horse blood agglutinin.	25/9	22/11	6/2	12/2	24/2	2/3*	4/3	7/3	10/3	14/3	17/3	21/3	25/3	28/3	1/4	4/4	8/4	16/4	21/4
		4.0	4.0	2.5	3.0	3.0	2.5	2.7	2.5	2.7	2.0	2.5	2.5	2.5	2.7	2.5	2.7	2.0	2.0	2.2
	Colon agglutinin.	5.0	5.0	5.0	5.0	5.0	5.4	5.4	4.0	5.0	2.9	5.0	5.0	5.0	5.9	5.4	6.3	5.4	5.4	5.9
20 and 21	Rabbit blood agglutinin.	3/3*	5/3	7/3	10/3	14/3	17/3	21/3	25/3	28/3	1/4									
		4.0	2.7	2.5	5.0	1.5	2.5	1.8	1.8	2.9	1.8									
	Colon agglutinin.	22.5	22.7	22.7	20.0	18.0	19.0	18.0	13.3	21.7	22.7									
	Horse blood agglutinin.	2.7	2.5	2.0	5.4	4.3	2.5	1.3	1.3	5.0	1.5									

KID NUMBER	TEST MADE FOR	KID NUMBER	KID'S SERUM									MILK						
7 and 8	Typhoid agglutinin.		21/2	23/2	25/2	28/2	3/3	11/3	16/3	21/3								
		7	0	1.7	1.6	1.7	1.7	1.4	1.3	1.3								
		8	0	1.7	1.6	1.4			0	2.0								
9 and 10	Typhoid agglutinin.		24/2	26/2	28/2							24/2	26/2	3/3				
		9	0	1.0	0							>2.5	0	0				
		10	0	0	0													
12 and 13	Horse blood agglutinin.		27/2	28/2	2/3	5/3	7/3	11/3	14/3	17/3	21/3	27/2	28/2	3/3				
		12	0	8.8	10.1	4.6	3.8		1.3	1.4	0	0	2.0	0				
		13	0	2.9	4.0	5.7	3.8	0.8	0.8	0	0							
18 and 19	Horse blood agglutinin.		2/3	4/3	7/3	10/3	14/3					2/3	4/3	7/3	10/3	14/3	17/3	21/3
		18	0	4.7	1.8	1.2	0					10.0	2.7	0.6	<0.5	0	0	0
		19	0	4.6	1.6	0.8	0											
	Colon agglutinin.	18	0	4.6	2.3	0	0					5.9	1.3	<1	<1	<1	1.3	0
		19	0	4.6	2.7	0	0											
20 and 21	Rabbit blood agglutinin.		3/3	5/3	7/3	10/3	14/3	17/3				3/3	5/3	7/3	10/3			
		20	0	0.2	0	0	0	0				5.4	0	0	0			
		21	0	1.7	0	0	0	0										
	Colon agglutinin.	20	0	5.9	3.5	1.9	1.9	0				5.0	1.3	1.4	0			
		21	0	9.7	3.7	3.7	3.3	0										
	Horse blood agglutinin.	20	0	2.4	1.0	0	0	0				5.4	1.3	<1	0			
		21	0	2.3	c. 1.	<1	0	0										

23 and 24	Colon agglutinin.	22/11	12/2	24/2	3/3*	5/3	7/3	11/3	17/3												3/3	5/3								3/3	5/3	7/3	11/3	17/3	
		13.9	13.9	14.4	14.4	17.0	19.4	22.4	22.5											23	0	8.1	†							27.5	6.8	1.3	0	0	
																				24	0	†													
	Horse blood agglutinin.	11.0	3.0	3.3	3.3	3.3	2.5	2.7	2.5											23	0	2.4								5.0	2.0	0	<0.5	0	
																				24	0														
	Rabbit blood agglutinin.	3.3	2.5	2.7	4.2	2.8	2.0	2.5	2.7											23	0	1.2								>5	1.4	<1	<0.5	<0.5	
																				24	0														
27 and 28	Colon agglutinin.	20/12	12/2	5/3*	7/3	10/3	13/3	16/3	28/3	4/4	11/4										5/3	7/3	10/3	13/3	16/3	28/3	4/4	10/4		5/3	7/3	10/3	13/3	17/3	
		8.3	6.6	9.0	8.3	8.3	6.6	5.7	6.6	8.7	8.7									27	0	5.1	4.0	3.0	2.5	0.5	0	0		25.0	2.5		0.5	0	
																				28	0.8	6.9	4.6	4.2	3.9	0.9	0.6	0							
	Horse blood agglutinin.	2.0	1.2	2.2	1.3	2.7	1.3	1.0	1.0	1.2	1.1									27	0	2.0	1.0	<1	0	0	0	0		8.3	0.67	0.5	0		
																				28	0	2.1	1.1	1.9	0	0	0	0							
	Typhoid agglutinin.	1.3	1.3	1.5	1.7	1.3	1.2	1.0	1.3	1.3										27		0.5	0	0	0	0	0	0		4.0		1.0	0		
																				28	0	1.6	<1	0	0	0	0	0							

Note: With the exception of 7 and 8, and 23 and 24, all kids and all milks were tested until about three months after birth. In order not to take up too much space, only the first tests are given; in the remainder there was no agglutinin.

found by Halban and Landsteiner in the mother's serum; and the titer of the milk is everywhere higher than that of the mother's serum, so that it is possible to speak of an accumulation of agglutinin.

In this connection, must be noted the researches of Wegelius (20) on the laws according to which antihemolysins and agglutinins are passively transmitted from the mother animal; he found that they were always transmitted, but that the antihemolysins were not transmitted with the milk and Morgenroth and Braun give as the result of their critical examination of this subject that an assimilation of the antibodies of the milk, it is true, has been proved in new-born young, but that it is not a regularly occurring phenomenon. It must, however, be borne in mind that this criticism applies to antibodies, which have either been produced artificially in the organism through immunization or have been directly mixed with the milk.

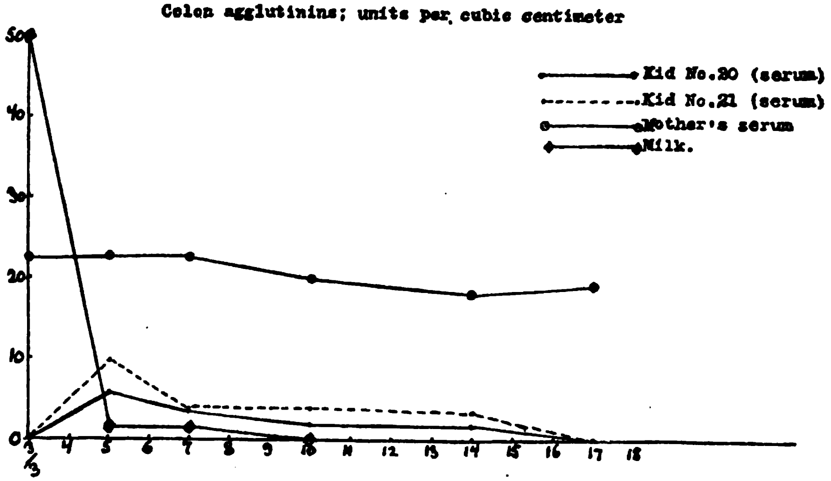


CHART 1

The figures quoted in table 1 are, in their reciprocal values, of such a homogeneous character that a graphic representation of one may serve as a paradigm of all (see chart 1).

In order to determine the transmission of the agglutinin through the milk, nursing experiments were undertaken; they were undertaken in such a manner that one kid of a pair of twins was removed from the mother before suckling and placed on boiled cow's milk, whereas the other was permitted to suckle; in another experiment raw cow's milk, free from agglutinin was used. The experiments are given below in table 2. Milk samples were taken from both the nipples of the mother animals, and it appears that the agglutinin disappears with varying rapidity in them; thus samples always ought to be taken from the nipple used.

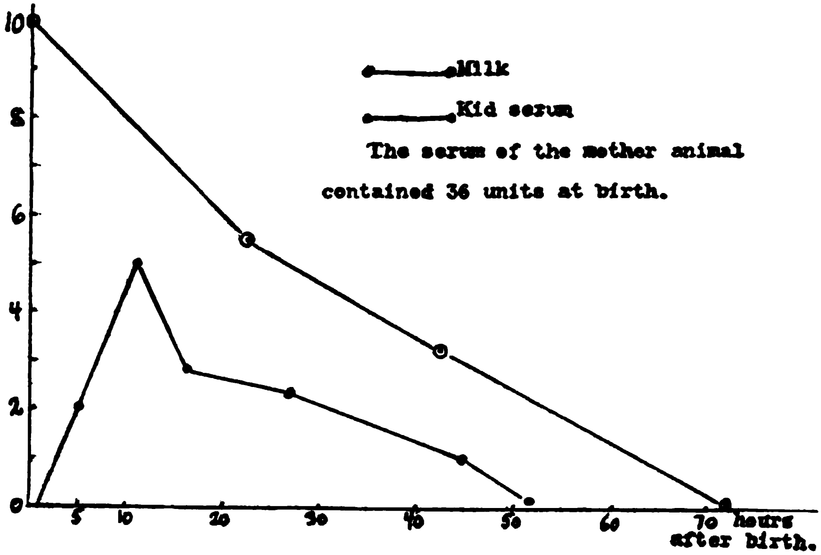
TABLE 2

	COLI-AGGLUTININ UNITS PER CUBIC CENTIMETER IN				
	Mother's serum	Serum of the kid removed from the mother and fed on cow's milk	Serum of the kid which remained with the mother	Milk from suckled nipple	Milk from unsuckled nipple
Experiment 1					
The kids born 3/19					
Samples taken 3/19	18.2	0	0	58.8	37.7
3/20	15.4	0	7.7	<5	12.5
3/21	20.0	0	5.9	—	10.0
3/22	15.4	0	5.5	2.5	7.7
3/24	15.4	0	4.4	2.2	7.7
Experiment 2					
The kids born 2/18		Fed only with raw cow's milk free of agglutinin			
Samples taken 2/18	7.1	0	0	15.4	
2/19	5.9	2.0	4.0	1.0	10.0
2/20	6.7	<1	2.8	1.0	5.4
2/22	7.6	<1	2.5	1.0	2.6
2/23	5.9	<1	1.3		

Experiment 1 shows the significance of the mother's milk in the occurrence of agglutinin in the blood of the kid; in experiment 2 it seems as if agglutinin had possibly been present in the cow's milk in a disguised form or in quantities too small to be measured; in any case the occurrence of agglutinin has been slight, considerably less than in the kid that remained with the mother.

According to tables 1 and 2 the greatest concentration of agglutinin in the serum of the kid occurs immediately after birth and in order more accurately to determine its place, test samples were taken with inter spaces of a few hours from a single kid and the milk of its mother. It now turned out, as is apparent from chart 2, that within a few hours after birth agglutinin can be demonstrated in the blood of the kid and that the height of the agglutinin curve lies about eleven hours after birth, where it reaches exactly half of the initial titer of the milk, which is interesting, in so far as a passive immunization through the digestive tract is otherwise difficult and at any rate uneconomical; thus, Wegelius, as mentioned above, could not prove the presence of antihemolysin in the blood of the kids, after feeding with milk containing antihemolysin. There is thus in all probability a difference between the assimilation of artificially transmitted antibodies and those which in a natural way have been produced in the blood of the mother animal. When the agglu-

Colon agglutinins ; units per cubic centimeter.



The serum of the mother animal contained 36 units at birth.

CHART 2

tinin has disappeared from the milk, it remains free of agglutinin (the longest regular experimental space was seventy-four days), and in all the samples taken I found only two with a faint agglutinating power.

In the blood of the kid the agglutinin reappears after a few months, probably, as indicated by several authors, in consequence of the colon-immunization from the digestive tract. As an example of this I present table 3; only the last of the kids put down in the latter is not to be found in table 1, but it was likewise examined immediately after birth and during the first three months of its life.

TABLE 3

Samples taken June 10 in the year of birth

KID NUMBER	DATE OF BIRTH	KIND OF AGGLUTININ	AGGLUTININ UNITS PER CUBIC CENTIMETER OF KID'S SERUM	AGGLUTININ UNITS PER CUBIC CENTIMETER OF MOTHER'S SERUM
7	2/21	Typhoid agglutinin	5.0	3.9
10	2/24	Typhoid agglutinin	1.2	4.0
13	2/27	Horse blood agglutinin	1.1	2.0
27	3/5	Horse blood agglutinin	0.7	2.8
27	3/5	Colon agglutinin	3.1	11.0
27	3/5	Typhoid agglutinin	1.3	2.0
36	3/12	Horse blood agglutinin	0.8	2.5
36	3/12	Rabbit blood agglutinin	0.7	2.0
36	3/12	Typhoid agglutinin	4.0	11.0

It appears from the above tables that there is a parallelism between the increase and decrease of the tested agglutinins in the kids' blood, and the circumstance that after having been absent for a considerable period they all reappear might suggest a mutual relationship; consequently an experiment was made to see whether it was possible through the injection of a bacterial agglutinin antigen to develop the production of a hemagglutinin and *vice versa*. The experiment was undertaken in the following manner: of two nearly six months old kids the one was injected with colon culture, the other with washed horseblood corpuscles, both subcutaneously with two injections of each antigen of 5 and 10 cc. respectively, on October 14 and 16. The graphic representation (chart 3) shows that both kids reacted by producing anti-horse blood as well as anti-colon agglutinin.

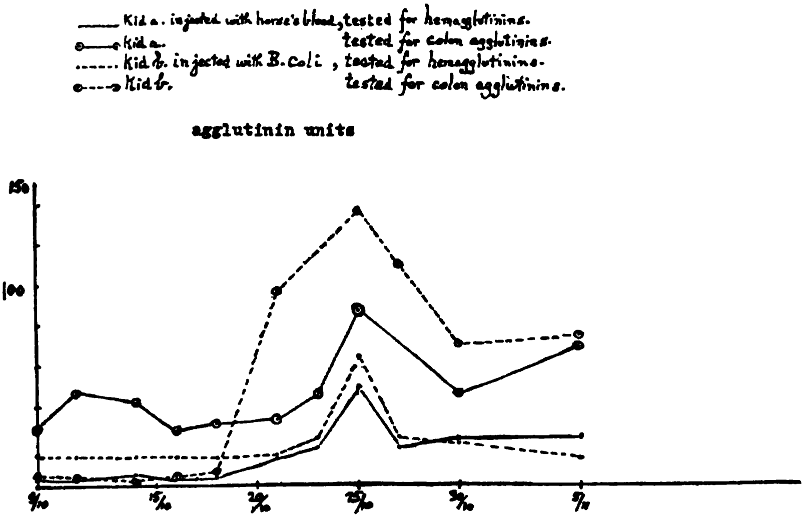


CHART 3

SUMMARY

1. By examining the transmission of the agglutinins normally occurring in the blood from mother to offspring in goats, I have only in one case of fourteen been able to prove their recurrence; in all the others the kids were born without agglutinin and probably derived it from the mother animal through the milk, in which it is to be found accumulated at parturition. From the milk as well as from the kid's serum it disappears in the course of a few days; then follows a period of a few months in which the blood of the kid is free of agglutinin, and then it appears again, probably in consequence of an immunization from the flora of the digestive tract. The research was concerned with coli and typhoid agglutinins, as well as agglutinin against rabbit and horse blood corpuscles.

2. In the blood of the kid there was in some cases more, in others less of the "normal antibodies" than in the blood of the mother animal, and only in one case the blood of the kid contained more than the colostrum, so that it is not possible to deduce any quantitative rule from these experiments.

3. In the colostrum the titer was higher than in the serum of the mother animal.

4. By nursing experiments it was shown in one case that the agglutinin is probably transmitted to the kid through the mother's milk, in another case the result was questionable.

5. The agglutinin maximum in the blood of the kid may occur as early as about 11 hours after birth.

6. By injections of horse blood corpuscles into kids it is possible to increase the agglutinating power as well against these corpuscles as against coli bacilli; *vice versa* by injection of coli bacilli the agglutinating power is increased also against horse blood corpuscles.

REFERENCES

(1) Gruenbaum, A. S.: Muench. med. Wochenschr., 1897, **44**, 330.
(2) Halban, J.: Wien. kl. Wochenschr., 1900, **13**, 545.
(3) Halban, J., and Landsteiner, K.: Muench. med. Wochenschr., 1902, **49**, 473.
(4) Schumacher, H.: Zeitschr. f. Hygiene u. Infektionskrankh. 1901.
(5) Schenk, F.: Monatschr. f. Geburtsh. u. Gynaekol., 1904, **19**, 159, 344, 568.
(6) Zubrzycki, J., and Wolfsgruber, R. Deutsche med. Wochenschr., 1913, **39**, 210.
(7) Kraus, R., and Loew, L.: Wien. klin. Wochenschr., 1899, **12**, 95.
(8) Kraus, R., and Lipschuetz, B.: Zeitschr. f. Hyg. u. Infektionskr., 1904, **46**, 49.
(9) Mueller, G.: Diss. Darmstadt, 1901.
(10) Park, W. H.: Proc. Soc. f. Exper. Biol. & Med., 1903, **1**, 19.
(11) Luedke, H.: Zentralbl. f. Bakt., Orig.. 1904, **37**, 288, 418.
(12) v. Eisler, M., and Sohma, M.: Wien kl. Wochenschr., 1908, **21**, 684.
(13) Klieneberger, C.: Deutsche Arch. f. kl. Med., 1907, **90**, 267.
(14) v. Fellenberg, R., and Doell, A.: Zeitschr. f. Geburtsch. u. Gynaekol., 1913, **75**, 285.
(15) Pfaundler, M.: Muench. med. Wochenschr., 1899, **46**, 473.
(16) Madsen, Th., and Jorgensen, A.: Festskrift. Statens Seruminstitut., 1902, no. VI.
(17) Madsen, Th., and Streng: Communicat. d. l'Institut therap. d. l'Etat Danois, **9**.

(18) MICHAELIS, L., AND DAVIDSOHN, H.: Biochem. Zeitschr., 1912, **47**, 59.
(19) WALBUM, L. E.: Biochem. Zeitschrift., 1914, **63**, 221.
(20) WEGELIUS, W.: Arch. f. Gynaekol., 1911, **94**, 265.
(21) MORGENROTH, J., AND BRAUN, H.: Die Vererbungsfrage in der Immunitaetslehre, Hbd. d. pathogenen Mikroorganismen V.
KOLLE AND WASSERMANN: 2 aufl., 1913, **2**, 1155.

STUDIES IN ANAPHYLAXIS

THE RELATION OF CERTAIN DRUGS TO THE ANAPHYLACTIC REACTION, AND THE BEARING THEREOF ON THE MECHANISM OF ANAPHYLACTIC SHOCK

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Comparatively few investigations have been recorded in the literature relative to the behavior of pharmacologic agents in the course of anaphylactic shock. Most of the studies of this kind have been made with a view of finding a substance which would relieve anaphylactic symptoms, and save the sensitized animal from the usually fatal results following the reinjection of the antigen in adequate doses. Thus, Besredka (1) found that guinea-pigs sensitized to ox serum recovered from a lethal dose of the antigen if the animals were under the influence of ether when the reinjection was made. Auer (2) has likewise shown the protecting influence of atropine in the course of anaphylactic shock. According to this author 3 mgm. of atropine injected into the subcutaneous tissues of sensitized guinea-pigs before the reinjection of the antigen exerted a favorable influence upon the outcome, as 3 out of 5 experimental animals thus treated were saved from immediate death. This effect of atropine, while interesting from the therapeutic standpoint, is also taken to throw light on the mechanism of the anaphylactic response. It is taken to mean that anaphylactic shock, in the guinea-pig at least, is due to a fatal broncho-constriction, which is peripheral in nature, since sectioning or degeneration of the vagi has no effect, while atropine partially prevents it. The results of Auer, however, did not find confirmation in the hands of Mita (3), who states that if the minimal lethal dose of the reinjected antigen be quantitatively determined per unit weight of animal, and if one makes sure that the full lethal dose be given in each instance, atropine exerts no prophylactic influence on the course of the anaphylactic reaction.

The work of Banzhaf and Famulener (4) may also be cited in this connection. These authors investigated the effects of chloral on the course of anaphylactic shock, and found that 100 to 150 mgm. of this drug injected intramuscularly into sensitized guinea-pigs saved 90 to 100 per cent of the experimental animals from anaphylactic shock.

Except for the experiments of Auer (loc. cit.) no work appears to have been done with pharmacologic substances with a view of a possible elucidation of the mechanism of anaphylactic shock.

The present studies deal in the main with the observation made some years ago of the peculiar influence of quinine on the course of the anaphylactic reaction. During the course of some experimental studies made on the antipyretic action of quinine in anaphylactic fever, it was found that this substance had no effects on sensitized rabbits if injected at various periods of the incubation interval. But if the drug was administered about a half hour before the reinjection of the foreign protein the animal had been sensitized to, a dose which ordinarily produced merely a rise in body temperature, and no other appreciable symptoms, now caused grave symptoms of anaphylactic shock, often terminating fatally. This observation appeared to have sufficient clinical importance, aside from the possibility that it might help to elucidate the mechanism of the anaphylactic response, and the matter was deemed worthy of a detailed study.

Rabbits and guinea-pigs were used throughout this research. The animals were in all instances actively sensitized. In some experiments horse serum[1] was used, while in most of the experiments ox serum was employed. Sensitization was effected in the guinea-pig with great regularity, by the intraperitoneal route, while the rabbits were usually sensitized by the subcutaneous route. Usually 2 cc. of ox serum injected subcutaneously in the rabbit is sufficient to render the animal anaphylactic within a period of fourteen to sixteen days. Occasionally rabbits are encountered that, for some unknown reason, are but incompletely sensitized by this method. In some experiments therefore three sensitizing doses, a subcutaneous, intraperitoneal and intravenous, were given at two to three day intervals, in the hope of effecting more complete sensitization. The anaphylactic dose was always injected intravenously; into the external jugular vein in the case of the guinea-pig, and into the marginal ear vein in the instance of the rabbit. The minimal anaphylactic dose of the specific antigen was determined in each series of experiments without and with the previous administration of quinine hydrochloride subcutaneously, in doses varying between 100 and 300 mgm. per kilo.

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[1] Normal horse serum of Parke, Davis and Company, containing 0.4 per cent cresol.

Tables 1 and 2 show the extent to which quinine influences the course of the anaphylactic response. In table 1 are given some of the results obtained with rabbits, while those obtained with guinea-pigs are presented in table 2. Series A of table 1 shows that while the minimal lethal dose of the reinjected antigen in sensitized non-treated animals is about 2 cc., that in animals previously treated with quinine is about 0.2 cc., giving a ratio of about 10:1. In series B and C of the same table, in which the rabbits were refractory to the specific protein, so that in the untreated animals at least 3 cc. of the serum were required to produce symptoms, animals previously treated with quinine succumbed to doses of 1 or 1.5 cc., thus presenting a ratio of 3:1 or over.

Reference to table 2 will likewise show a marked difference in the reaction to the reinjected antigen between normal sensitized animals, and those previously treated with quinine. Thus the minimal lethal dose of the reinjected antigen in guinea-pigs sensitized to horse serum is 0.2 cc. per 100 grams of body weight, while in those treated with 100 mgm. per kilo of quinine hydrochloride subcutaneously the dose is 0.075 cc. per 100 grams series A.B). Similarly non-treated animals sensitized to ox serum succumb to 0.03 to 0.04 cc. per 100 grams thereof, while animals treated with quinine die in anaphylactic shock from doses of

TABLE 1

Toxicity of reinjected antigen in sensitized rabbits as modified by quinine

	NO QUININE					QUININE HCl SUBCUTANEOUSLY					
Series	Experiment number	Sensitizing dose	Incubation period	Reinjected dose	Result	Experiment number	Sensitizing dose	Incubation period	Quinine HCl	Reinjected dose	Result
		cc.	*days*	*cc. per kgm.*			*cc.*	*days*	*mgm. per kgm.*	*cc. per kgm.*	
A	21	2.0	16	0.1	No symptoms	25	2.0	16	200	0.1	Symptoms
	22	2.0	16	0.5	No symptoms	26	2.0	16	200	0.1	Symptoms
	23	2.0	16	1.0	No symptoms	27	2.0	16	300	0.2	Died in several hours
	24	2.0	16	2.0	Died in 2 minutes	28	2.0	16	300	0.2	Died in several hours
	29	2.0	16	2.0	Symptoms	31	2.0	17	300	0.2	Died in several hours
	30	2.0	16	2.0	Died in several hours	32	2.0	17	300	0.4	Died in 1 minute
	34	2.0	17	2.0	Died in several hours						
B	37	1.0	20	2.0	No symptoms	36	1.0	20	300	2.0	Died in 2 minutes
	38	1.0	20	3.0	Died in 1 minute	39	1.0	20	300	1.0	Symptoms
	41	1.0	20	2.5	No symptoms	40	1.0	20	300	1.5	Died in several hours
	44	1.0	21	3.0	No symptoms	42	1.0	21	250	2.0	Died in several hours
	45	1.0	21	3.5	Symptoms						
	46	1.0	21	3.0	Symptoms						
C	99	3.0	15	2.0	Severe symptoms	103	3.0	20	200	2.0	Died in 4 minutes
	100	3.0	15	3.0	Severe symptoms	104	3.0	20	250	2.0	Died in 1 hour
	102	3.0	19	1.5	Symptoms	106	3.0	20	200	1.0	Severe symptoms
						107	3.0	20	200	1.0	Died in several hours
						108	3.0	20	200	0.5	Severe symptoms

TABLE 2

Toxicity of reinjected antigen in guinea-pigs as modified by quinine

	NO QUININE					QUININE HCl, 100 MILLIGRAMS PER KILOGRAM SUBCUTANEOUSLY				
Series	Experiment number	Sensitising dose	Incubation period	Reinjected dose	Result	Experiment number	Sensitising dose	Incubation period	Reinjected dose	Result
		cc.	*days*	*cc. per 100 grams*			*cc.*	*days*	*cc. per 100 grams*	
A H. S.	22	1.0	14	0.20	No symptoms	25	1.0	14	0.200	Died in 2 minutes
	23	1.0	14	0.10	No symptoms	27	1.0	14	0.100	Died in 9 minutes
	24	1.0	14	0.30	Died in 1 minute	31	0.5	14	0.010	No symptoms
	28	1.0	14	0.30	Symptoms					
	29	0.5	14	0.01	Mild symptoms					
	30	0.5	14	0.10	Mild symptoms					
B H. S.	33	1.0	21	0.20	Died in 35 minutes	39	1.0	21	0.075	Died in 10 minutes
	34	1.0	21	0.25	Died in 3 minutes	50	0.5	21	0.100	Died in 1 minute
	48	0.5	21	0.15	Mild symptoms					
	49	0.5	21	0.10	Mild symptoms					
C O. S.	74	0.1	16	0.20	Died in 2 minutes	81	0.1	16	0.010	Died in 4 minutes
	75	0.1	16	0.20	Died in 3 minutes	82	0.1	16	0.003	Symptoms
	76	0.1	16	0.10	Died in 3 minutes	83	0.1	16	0.005	Symptoms
	77	0.1	16	0.10	Died in 3 minutes	98	0.5	15	0.008	Died in 4 minutes
	78	0.1	16	0.05	Died in 4 minutes	99	0.5	15	0.005	Symptoms
	79	0.1	16	0.01	No symptoms	100	0.5	15	0.006	Symptoms
	80	0.1	16	0.03	Died in 3 minutes	101	0.5	15	0.007	Symptoms
	84	0.5	12	0.05	Died in 4 minutes					
	85	0.5	12	0.02	Symptoms					
	86	0.5	12	0.03	Died in 4 minutes					
D O. S.	116	0.2	20	0.04	Died in 4 minutes	119	0.2	20	0.020	Died in 3 minutes
	117	0.2	20	0.03	Symptoms	120	0.2	20	0.010	Died in 3 minutes
						121	0.2	20	0.008	Died in 3 minutes

0.008 cc. per 100 grams, or less (series C and D). Thus quinine increases the susceptibility of sensitized rabbits and guinea-pigs from 3 to 10 times to the effects of the reinjected antigen.

The incubation period has not been found to be materially altered by quinine. That is to say, if the incubation period in a given instance was too short for sensitization to be effected, and no symptoms were produced by the reinjected antigen, the previous administration of quinine did not alter the reaction. In some experiments on the rabbit to determine this point, it was found that not until eight or nine days have elapsed since the primary injection of the foreign protein did quinine influence the reaction to the reinjected antigen. This also appears to be the minimal period for effecting sensitization.

The question that naturally presented itself was, what is the cause of the peculiar influence of quinine on the course of the anaphylactic reaction? Why do animals sensitized to a foreign protein react with such a degree of hypersensitiveness to the reinjected antigen if previously treated with quinine, even in doses too small to show any definite symptoms, while in non-sensitized animals treated with large doses of quinine the injection of a foreign serum produces no effects whatever?

Experiments were undertaken in the hope of finding an answer to the above questions on the basis of the prevailing theories of the mechanism of anaphylactic shock. In the main there are two theories for the explanation of this phenomenon. Briefly stated, they are the humoral or chemical theory, and the cellular or physical theory. The former assumes that the anaphylactic reaction is an intoxication brought about by toxic substances elaborated in the blood stream of the sensitized animal as a result of interaction between antigen and antibody, it being held that the latter possesses the nature of an enzyme. The cellular theory assumes that the reaction is due to attached cellular antibody binding the antigenic substance.

As stated, in terms of the humoral theory anaphylactic shock is an intoxication, the poison being produced by the digesting action of the specific proteolytic enzyme on the reinjected antigen (Vaughan 5). More recently the view has been expressed that it is the proteins of the serum of the sensitized animal that are split rather than those of the reinjected antigen (Jobling, Petersen, and Eggstein 6). The last named authors assume that the proteolysis is caused by a deficiency of the antiferments in the serum of the sensitized animal.

Since quinine is known to have a powerful influence on all ferment action, it seemed probable that an investigation into the influence of this substance on the rate of proteolysis resulting from the interaction of sensitized serum and specific antigen might not only explain the altered susceptibility of sensitized, quinine-treated animals to the reinjected antigen, but it might also add further evidence in favor of the humoral conception of anaphylaxis. With this aim in view rabbits and guinea-pigs were sensitized in the usual manner to ox serum. After a sufficiently long incubation period the animals were bled, the sera collected, and definite amounts thereof were treated with definite amounts of the antigenic serum, and were incubated for some hours at 38°C., without or with the addition of known amounts of quinine. The degree of proteolysis in the various specimens was determined by a chemical analysis of the non-coagulable nitrogen in the respective sera, and in the mixtures after incubation.

The method employed for the determination of the non-coagulable nitrogen was essentially that of Folin and Wu (7), with some slight modifications which became necessary in the course of this work. Briefly the method employed was as follows. One cubic centimeter of the respective sera or mixtures thereof was pipetted into a large pyrex glass test tube, and the coagulable proteins were precipitated by the addition of 1 cc. of 10 per cent acetic acid in 20 per cent sodium chloride solution, and gently boiling this for about five minutes. This deviation from Folin's method was necessary since it was desirable to look not only for the non-protein nitrogen, but also for that of the higher split products. The boiled mixture was filtered, the precipitate washed with boiling distilled water, and the filtrate made up to a definite volume. An accurately measured portion thereof was used for digestion in pyrex glass test tubes. Here another deviation from the Folin method had to be made. The digestion could not be successfully carried out by means of the phosphoric-sulphuric acid mixture recommended by Folin; presumably the acetic acid interfered. The digestion mixture employed, therefore, consisted of sulphuric acid, copper sulphate, and sodium sulphate, as recommended by Peters (8). The digested mixture was allowed to cool, about 10 cc. of water added, then 2 cc. of 5 per cent Rochelle salt to prevent turbidity (8), 10 per cent sodium hydrate added to slight alkalinity, 5 cc. of the undiluted Folin-Nessler reagent, and water to make a definite volume. The amount of nitrogen was determined by comparing the color of this solution in a Duboscq colorimeter with that of a definite amount of nitrogen in the form of ammonium sulphate, treated similarly in every respect, except for the digestion, which was omitted.

The results obtained in these experiments are given in table 3. Most of the experiments were made in duplicates. The amounts of sensitized serum and of the antigenic serum used are indicated in their respective columns. Wherever quinine was used the amount indicated as the hydrochloride was dissolved in 1 cc. of water. The same volume of water was also added to those mixtures in which no quinine was employed, except in experiment 26, in which no water was added. In the "found" column the figures represent the amounts of nitrogen per cubic centimeter of the mixture, actually obtained by analysis. The figures given in the "calculated" column represent the amount of nitrogen per cubic centimeter of the mixture, theoretically calculated, including the theoretical amount of nitrogen in the quinine, wherever this was added.[2] To illustrate: In experiment 12, 2 cc. of sensitized rabbit's serum containing 1.12 mgm. of nitrogen per cubic centimeter were mixed with 1 cc. of ox serum containing 0.52 mgm. of nitrogen per cubic centimeter. One cubic centimeter of water containing 5 mgm. of quinine hydrochloride was added to this mixture, and the whole was incubated at 38°C. for four hours. One cubic centimeter of this mixture after incubation was found on analysis to contain 0.77 mgm. of non-coagulable nitrogen. The calculated amount of nitrogen for 1 cc. of this mixture is $2\times1.12+0.52+0.3$ (the theoretical equivalent of nitrogen in 5 mgm. of quinine $HCl.3H_2O$), and the whole divided by 4, giving 0.77 mgm.

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[2] A blank experiment was made to determine the nitrogen content of the quinine used, and it was found to be practically identical with the theoretical figure.

In series A of this table the sensitized serum was obtained by exsanguination under ether of a rabbit that had been sensitized to ox serum in the usual manner. The incubation period was sixteen days. This serum contained 1.12 mgm. of non-coagulable nitrogen per cubic centimeter. The antigen used in this and in the next series contained 0.52 mgm. per cubic centimeter. The sensitized serum used in series B was obtained from a rabbit similarly treated, but bled without anesthesia. This serum contained 1.40 mgm. of nitrogen per cubic centimeter. In series C the sensitized serum was obtained from another rabbit, similarly treated, and bled under ether. The non-coagulable nitrogen content of this serum was 1.75, while that of the antigen used in this and in

TABLE 3

Non-coagulable nitrogen of sensitized serum incubated with antigen and quinine

SERIES	EXPERIMENT NUMBER	SENSITIZED SERUM	ANTIGEN, OX SERUM	QUININE HYDROCHLORIDE	INCUBATED	NON-COAGULABLE NITROGEN PER CUBIC CENTIMETER	
						Found	Calculated
		cc.	*cc.*	*mgm.*	*hours*	*mgm.*	*mgm.*
A	11	2	1	0	4	0.70	0.69
	12	2	1	5	4	0.77	0.77
	13	1	2	0	4	0.61	0.54
	14	1	2	5	4	0.77	0.62
	15	5	15	0	4	0.60	0.64
	16	5	15	10	4	0.65	0.66
	17	5	15	0	0	0.57	0.64
B	20	3	2	0	3	0.80	0.87
	21	3	2	5	3	1.00	0.92
C	22	5	5	0	4	0.96	1.16
	23	5	5	5	4	1.00	1.18
D	24	2	2	0	4	0.80	0.77
	25	2	2	5	4	0.87	0.83
E	26	2	1	0	4	0.58	0.56
	27	1	2	1	4	0.40	0.51

the subsequent series was 0.80 mgm. per cubic centimeter. The sensitized sera of series D and E were obtained from pooled blood of three guinea-pigs each, that had been sensitized to ox serum, and bled without anesthesia after an incubation period of thirteen and fifteen days respectively. The sensitized serum of series D contained 1.12 mgm. of non-coagulable nitrogen per cubic centimeter, and that of series E contained 0.44 mgm. per cubic centimeter.

It will be seen that the amounts of non-coagulable nitrogen found in the various mixtures approach very closely the calculated amounts for those mixtures. In some of the experiments (13, 14, and 21) the figures are somewhat higher, while in several experiments they are decidedly lower than the calculated amounts. In some of the mixtures in which the "found" figures are lower than in the corresponding "calculated" figures, precipitates had formed on incubation, the mixtures were centrifugalized, and the clear serum was used for analysis.

De Kruif and German (9) in studying the relation between the production of anaphylatoxin under the influence of agar, and serum autolysis as measured by the aliphatic amino acid nitrogen in the serum, also observed a diminution of the latter upon incubation with agar. They ascribe this diminution to adsorption of some of the amino acids by the agar. Their attempts to recover this supposedly adsorbed amino acid was not successful. A more plausible explanation, for these experiments at least, would seem to be that some of the non-coagulable nitrogen might have been thrown down in the precipitate, perhaps in some chemical combination.

The present results do not give evidence of proteolysis taking place as a result of interaction between sensitized serum and antigen, whether such mixtures be treated with quinine or not. The altered susceptibility of sensitized animals to reinjected antigen when under the influence of quinine, is therefore not explainable on the basis of the action of quinine on ferments. Nor do these experiments lend support to the theory that proteolysis is an essential factor in the causation of anaphylactic shock.

The evidence heretofore adduced in favor of proteolysis resulting from the interaction of antigen and antibody has been rather contradictory. Zunz and György (10) determined the amino acid nitrogen of the serum of guinea-pigs, rabbits and dogs that had been sensitized to ox serum, protalbumose, and heteroalbumose. They incubated the sensitized sera with the respective specific proteins at different stages of the incubation period, and determined the aliphatic amino acid nitrogen in the incubated mixtures. In some cases they obtained an increase, in others a decrease. The results, indeed, were so variable in the various sets of their experiments that the authors are not willing to draw any conclusions, and state that the changes in the proteolytic power of sensitized blood do not suffice to explain the phenomenon of anaphylaxis. Auer and Van Slyke (11) determined the amino acid nitrogen content of the lungs in sensitized guinea-pigs before and during shock, and found no difference. De Kruif and German (loc. cit.) failed to get an increase of the amino acid nitrogen in the serum of rabbits sensitized to egg white, and incubated with the latter at 38°C. Jobling, Petersen and Eggstein (loc. cit.) determined the non-coagulable nitrogen in sensitized dog's serum before and during shock, and found an increase in the latter. They believe the proteins of the animal's own serum to undergo proteolysis owing to disturbed ferment antiferment equilibrium produced by the antigen. There are undoubtedly other factors than anaphylactic shock that may alter the amount of non-coagulable nitrogen in the blood, and it is not impossible that the changes noted by these authors may have been the effect rather than the cause of shock. Manwaring, Kusama, and Crowe (12) in an investigation of the fate of the foreign protein in acute anaphylactic shock conclude that there is no evidence of a measurable destruction of the foreign protein by the blood serum of the sensitized animal.

Failing to find an explanation for the peculiar influence of quinine on the course of anaphylactic shock on the basis of the humoral theory, experiments were next undertaken in a different direction. Coca (13) has recently shown that upon perfusion of the pulmonary vessels of sensitized rabbits with Locke's solution containing some of the antigen, there is a marked obstruction to the flow of the perfusion fluid, for the pressure required to initiate a flow of the perfusion fluid under these conditions was found much higher than when such a fluid was perfused through the vessels of a normal animal. Coca regards anaphylactic shock in the rabbit as a cellular reaction to the antigen, especially of the pulmonary arterioles, resulting in pulmonary obstruction, and a consequent dilatation of the heart. I have been able to confirm the above observation, by means of a somewhat different technique.

TABLE 4

Effect of antigen and of quinine on the caliber of the pulmonary vessels in normal and sensitized rabbits

EXPERIMENT NUMBER	ANIMAL	WEIGHT	HYDROSTATIC PRESSURE; RINGER-LOCKE SOLUTION	OUTFLOW IN 3 MINUTES AVERAGE	OX SERUM	QUININE HCl	OUTFLOW IN 3' MINUTES	REMARKS
		kgm.	*cc.*	*cc.*			*cc.*	
1	Normal	2.5	40	76	1:300	0	70	
					1:40	0	72	
2	Sensitized	1.7	25	40	0	1:10000	44	
						1:1000	52	
					1:400	0	14	Discontinued
3	Sensitized	1.8	35	82	0	1:3500	80	
					0	1:1250	90	
					1:150	0	75	
					1:40	0	30	Discontinued
4	Sensitized	2.5	45	105	1:200	0	90	
					1:40	0	58	Discontinued

Briefly my experiments were as follows. Sensitized rabbits were bled from the carotid artery, a cannula was inserted into the left pulmonary artery, and one into the left auricle. Perfusion of Ringer-Locke solution at 38°C. under a constant hydrostatic pressure was immediately instituted, while the lungs were rhythmically inflated about 25 times per minute. When the return of the perfusion fluid remained constant, a definite amount of the antigen (ox serum) was added and the rate of outflow noted. Since serum is known to have an effect on smooth muscle, control experiments were made on normal animals and it was found that as high a concentration as 1:40 of ox serum in Ringer-Locke solution had no influence on the rate of flow of the perfusion fluid through the pulmonary vessels. Higher concentrations were not tried. If such a solution is perfused through the pulmonary vessels of rabbits sensitized to ox serum, a considerable diminution of the rate of outflow occurred, as will appear from the subjoined table 4.

The effects of quinine on smooth muscle in certain situations is well known, and need not be discussed here. It was thought that quinine might exert an action on the smooth musculature of the pulmonary vessels in the same direction as the specific antigen in the sensitized animals and thus explain the augmented susceptibility of sensitized animals to the specific foreign protein when under the influence of this drug. Quinine was found, however, to have no appreciable effect on the caliber of the pulmonary vessels when perfused through them in such concentration as might attain in the blood stream. In higher concentrations it appears to relax the pulmonary vessels, rather than constrict them. The influence that quinine has on the course of anaphylactic shock cannot therefore be referred to the altered pulmonary circulation caused by the foreign protein in the sensitized animal.

THE RELATION OF HISTAMINE (β-IMINAZOLYLETHYLAMINE) TO ANAPHYLAXIS

In a recent important contribution on the occurrence of histamine in various animal tissues, and its probable rôle in a variety of physiological and pathological processes, Abel and Kubota (14) suggest that this substance may also play an important part in the mechanism of anaphylaxis. They suggest that this may be the hypothetical "poison" producing the symptoms of anaphylactic shock. They indeed, express the view that histamine may be identical with Vaughan's toxic cleavage product obtained from various proteins. Vaughan has long called attention to the close similarity between the physiological action of histamine as described by Dale and his collaborators, and the physiologic behavior of his protein poison (5). Thus Vaughan pointed out that the fatal dose of his purified "poison" is practically the same as that of histamine for the guinea-pig. Both substances cause a lowering of the blood pressure in the dog. The striking broncho-constriction, and lung immobilization in the guinea-pig in anaphylactic shock and the similar picture in histamine poisoning is a familiar thing. However, such superficial similarities in the symptoms cannot be taken as final proof of the identity of the two conditions producing them. In order to determine more definitely to what extent histamine is related to anaphylactic shock, experiments were undertaken with this substance to see whether it is capable of producing phenomena generally regarded characteristic of anaphylactic shock. It is assumed in these experiments that if anaphylactic shock is associated with proteolysis with histamine as one of its products, then the characteristic phenomena occurring during shock should also be produced by histamine.

The experiments described below relate to the effects of histamine on body temperature; its influence on the coagulability of the blood; its relation to quinine; and its relation to desensitization or antianaphylaxis.

a. Effects of histamine on body temperature

The effects on body temperature produced by the injection of a foreign protein into an animal sensitized thereto have been described and studied by Pfeiffer (15), Friedberger and his collaborators (16), Hashimoto (17), Smith (18), and others. It has been shown that rabbits sensitized to ox serum and reinjected therewith after the incubation period, will have a rise in body temperature if the dose is small, and a fall in body temperature if the dose is large but sublethal (18). To determine whether histamine produces similar changes in body temperature, rabbits were injected intravenously with this substance in varying doses, and their temperature taken for some time thereafter. The minimal lethal dose of histamine on intravenous injection in the rabbit was found to be 0.4 mgm. per kilo. When histamine was injected in amounts from 0.01 to 0.3 mgm. per kilo, in single or repeated doses, no appreciable and constant effect was produced on the temperature of the rabbit. With the smaller doses no symptoms whatever could be elicited, while with larger doses (0.1 to 0.3 mgm. per kilo) there was considerable respiratory distress, contraction of the pupils, muscular weakness and convulsions, followed by recovery in a few minutes. No definite effect on body temperature could be elicited even with these massive doses.

b. The influence of histamine on the coagulability of the blood

The diminished coagulability of the bood of animals in anaphylactic shock is well known. This phenomenon of anaphylactic shock is most pronounced in the dog, to a less extent in the rabbit and the guinea-pig. The coagulation time of the blood in shocked rabbits is however sufficiently prolonged from the normal to make this animal of service in comparative studies of this phase of anaphylaxis. As a result of several experiments in which the same procedure was employed to determine the comparative coagulability of the blood in rabbits the following conclusions have been arrived at. The blood of normal rabbits coagulates in from five to seven minutes. The blood of rabbits in anaphylactic shock coagulates in from fifteen to twenty minutes. The coagulation time of the blood of rabbits shocked with histamine does not materially differ from the normal; indeed, it appears to be somewhat shorter than the normal.

c. The relation of quinine to histamine

It was shown in the earlier part of this paper that quinine profoundly influences the susceptibility of sensitized animals to the specific protein. If anaphylactic shock is to be interpreted in terms of the liberated histamine as a result of proteolysis or any other mechanism, it seems reasonable to suppose that quinine should equally alter the susceptibility of animals to this substance. In other words, there should be a considerable degree of synnergy between quinine and histamine. Accordingly, normal and sensitized rabbits and guinea-pigs were treated with quinine hydrochloride subcutaneously in doses of from 100 to 300 mgm. per kilo, and in about a half hour were injected intravenously with histamine in varying doses. Quinine was not found to have any effect on the course of histamine intoxication. In fact, it was frequently noted that a toxic but not lethal dose of histamine injected into rabbits previously treated with quinine produced milder symptoms than a corresponding dose of histamine given alone.

Of course, it may be argued that quinine causes the hypothetical poison (histamine) to be produced in greater amounts or at a greater rate, without necessarily having to act synnergistically with it. This is not probable, however, in the light of the results obtained from the experiments on the non-coagulable nitrogen in various mixtures of sensitized and antigenic sera.

d. The relation of histamine to antianaphylaxis

It is a well known fact that if a minute dose of a foreign protein be injected into an animal sensitized thereto, a state of antianaphylaxis is induced, the animal becomes desensitized, and withstands a lethal or several times the lethal dose of the foreign protein. This phenomenon is so constant that it is often regarded as a criterion in the determination whether or not a given anaphylactoid reaction belongs to the class of true anaphylaxis. Thus Besredka states that peptone injected into guinea-pigs sensitized to horse serum does not protect them from this foreign protein; and he uses this argument in controverting the contention made by some authors that peptone shock is identical with anaphylactic shock (1).

It appeared justifiable to assume that if anaphylactic shock is caused by histamine liberated by proteolysis, and since the preliminary injection of a small dose of the specific foreign protein protects the sensitized animal from larger doses of this protein, then the primary injection of a small amount of histamine should equally protect the sensitized animal from a lethal dose of the foreign protein. The converse, that is to say, the protecting action of a desensitizing dose of a specific foreign protein in a sensitized animal against a lethal dose of histamine is not essential, for it may be assumed that desensitization in some manner inhibits further proteolysis, but cannot protect the animal from a lethal dose of the preformed poison.

Several experiments were made on rabbits and guinea-pigs to see whether preliminary injections of histamine into sensitized animals could protect them from the effects of the specific foreign protein, and whether desensitization would influence the toxicity of histamine. The results were entirely negative. Neither did the preliminary injection of histamine affect the course of anaphylactic shock, nor did sensitization or desensitization in any manner influence the toxicity of histamine.

It may be added that several experiments were also made on guinea-pigs to determine whether a given fraction of the lethal dose of histamine could replace a corresponding fraction of the lethal dose of the specific foreign protein. This was found to be the case. In one series of guinea-pigs sensitized to ox serum, the minimal lethal dose of the antigen was found to be 0.03 cc. per 100 grams. The minimal lethal dose of histamine was determined at 0.02 mgm. per 100 grams. Fifty per cent of the minimal lethal dose of each administered at the same time proved fatal. Indeed, 50 per cent of the one combined with 35 per cent of the other proved fatal in some experiments. It would seem, therefore, that the point of attack of histamine and of the toxic factor in anaphylaxis is the same in the guinea-pig. This point was not investigated in the rabbit. It is not unlikely that a similar synnergy between histamine and the specific foreign protein might exist in this animal too, for Dale and Laidlaw (19) have shown that the cause of death from histamine in the rabbit is due to pulmonary obstruction leading to acute dilatation of the right heart, and a similar mechanism for anaphylactic shock in the rabbit is suggested by the experiments of Coca (loc. cit.), which I have been able to confirm.

SUMMARY AND CONCLUSIONS

Guinea-pigs and rabbits sensitized to ox serum or horse serum, and treated subcutaneously with moderate doses of quinine preceding the reinjection of the specific antigen, have their susceptibility increased from 3 to 10 times to the specific protein, as compared with control sensitized animals.

No appreciable degree of proteolysis could be demonstrated to occur in vitro by treating sensitized serum with the specific antigen, whether incubated alone or with quinine. The augmented susceptibility of sensitized animals to the specific protein when under the influence of quinine cannot, therefore, be referred to the well known action of this drug on ferments.

Quinine added to Ringer-Locke solution perfused through the pulmonary vessels of sensitized or normal rabbits does not cause any noticeable constriction of these vessels. Specific foreign protein added to Ringer-Locke solution perfused through the pulmonary vessels of sensitized rabbits produces pulmonary obstruction to a marked degree. The altered susceptibility of sensitized animals to the foreign protein produced by quinine cannot be referred to any synnergy between quinine and the anaphylactic process on the pulmonary circulation.

It has been suggested that histamine might be the causative factor of anaphylactic shock. On closer investigation it does not appear that histamine is identical with the anaphylactic process, for the following reasons:

a. Histamine does not produce in animals the temperature reactions observed in anaphylaxis.

b. Histamine does not alter the coagulability of the blood, as is noted in anaphylactic shock.

c. Quinine alters markedly the course of the anaphylactic reaction, by augmenting the susceptibility of sensitized animals to the foreign protein; it has no harmful effect on the course of intoxication with histamine.

d. There is no relation between histamine and antianaphylaxis. Neither does desensitization influence the toxicity of histamine, nor does the preliminary treatment with histamine alter the lethal dose of the specific foreign protein in sensitized animals.

A synnergetic relation is shown to exist between histamine and the specific foreign protein in sensitized guinea-pigs. This is probably best explained on the assumption that some points of attack of histamine and of the anaphylactic process are identical.

REFERENCES

(1) **Besredka**; Anaphylaxis and Antianaphylaxis. 1919, Mosby & Co.
(2) **Auer**: Jour. Exp. Med., 1910, **12**, 638.
(3) **Mita**: Zt. f. Immunitaetsf., 1911, **11**, 501.
(4) **Banzhaf and Famulener**: Jour. Inf. Dis., 1910, **7**, 577.
(5) **Vaughan**: Protein Split Products. 1913, Lea and Febiger, Jour. Am. Med. Assn., 1914, **62**, 583.
(6) **Jobling, Petersen and Eggstein**: Jour. Exp. Med., 1915, **22**, 401.
(7) **Folin and Wu**: Jour. Biol. Chem., 1919, **38**, 81.
(8) **Peters**: Ibid., 1919, **39**, 285.
(9) **De Kruif and German**: Jour. Inf. Dis., 1917, **20**, 833.
(10) **Zunz and György**: Zt. f. Immunitaetsf. Orig., 1914–15, **23**, 402; Ibid., 1913, **17**, 241; 265; 279.
(11) **Auer and Van Slyke**: Jour. Exp. Med., 1913, **18**, 210.
(12) **Manwaring, Kusama, and Crowe**: Jour. Immunol., 1917, **2**, 511.
(13) **Coca**: Ibid., 1919, **4**, 219.
(14) **Abel and Kubota**: Jour. Pharm. and Exp. Ther., 1919, **13**, 243.
(15) **Pfeiffer**: Wien. Klin. Woch., 1909, **22**, 14.
(16) **Friedberger and Mita**: Zt. f. Immunitaetsf., 1911, **10**, 216.
(17) **Hashimoto**: Arch. f. Exp. Path. u. Pharmak., 1914, **78**, 370.
(18) **Smith**: Jour. Lab. Clin. Med., 1915–16, **1**, 902.
(19) **Dale and Laidlaw**: Jour. Physiol., 1910, **41**, 318; Ibid, 1911, **43**, 182.

THE ANTIGENIC PROPERTIES OF HEMOCYANIN[1]

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Comparatively little is known concerning the chemistry of hemocyanin, the copper-protein compound found in the circulating fluid of many Crustacea, Mollusca and other invertebrates. Functionally it appears to play the rôle of oxygen carrier, being in this respect analogous to hemoglobin. Hemocyanin unlike hemoglobin is not confined to particular cells but appears free in the circulating fluid of which in many of these animals it forms the chief protein component.

Concerning its chemical nature no unanimity exists since different workers have obtained results with hemocyanin obtained from different sources which are at variance to an extent greater than usually found in proteins prepared from allied species (1) Henze (2) prepared crystalline hemocyanin from the circulating fluid of the octopus (*Octopus vulgaris*) by precipitation with ammonium sulphate according to the procedure of Hopkins and Pinkus. On dialysis a 3 per cent solution of hemocyanin was obtained. On addition of acid, part, but not all of the copper could be split off, but splitting in the sense that hemoglobin is split to yield a protein and a chromogenic substance did not take place. Henze believes, from a study of its reactions, that hemocyanin is a copper albuminate.

Alsberg and Clark (3) carefully went over the ground covered by Henze but used the blood of *Limulus polyphemus* as a source of hemocyanin. Not only did their product differ from Henze's with respect to the concentration of ammonium sulphate re-

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quired for precipitation, it behaving in this respect like a globulin, but they also found a marked difference in elementary composition and a decidedly lower copper content. Like Henze, they were unable to obtain from Limulus hemocyanin a substance analogous to hematin. It appears that the hemocyanins from these two animals are distinctly different and this conclusion is supported by a comparison of the stability of the oxygen compounds, hemocyanin from Limulus being but little dissociated (4) under diminished pressure while that from the octopus gives up its oxygen more readily. Dhéré (5) has also noted differences in the copper content and oxygen capacity of the blood of various invertebrates.

Immunological experiments with pure proteins have shown that antigenic property is intimately connected with their chemical structure. Thus amino acids, polypeptides, protamines, histones, certain proteoses, and gelatin fail to give rise to demonstratable immune bodies when repeatedly injected into animals. Gliadin from wheat and hordein from barley resemble each other in their chemical makeup and are closely related immunologically (6). The immunological method can at times be used for the purpose of gaining further insight into the chemical makeup of a particular protein. Gross chemical analyses yield but little information since they do not take into consideration the stereochemical configuration in the protein molecule, nor is it at times possible by chemical methods to demonstrate differences in closely related proteins which by immunological methods can readily be shown. However it is of interest in this connection to mention that a high histidin content is characteristic of both hemoglobin and hemocyanin (7).

It has been shown that neither globin (8), hematin or their combination, as in hemoglobin (9), is antigenic. The inability of globin to give rise to immune bodies when injected repeatedly into animals cannot be attributed to lack of aromatic amino acids (10) and in this respect differs from gelatin, which, according to an hypothesis (11), is non-antigenic because of this deficiency.

It appeared to us that the immunological method might be used to establish the relationship of the hemocyanins derived from different sources and also to throw further light on its chemical makeup. Nuttall (12) and von Dungern (13) were able to immunize rabbits against the circulating fluid of cephalopods and other invertebrates and von Dungern and Cohnheim (14) found that copper was contained in the specific precipitate obtained with octopus serum, indicating that part of the precipitate consisted of hemocyanin.[2] While this appears to indicate that hemocyanin is antigenic, it does not exclude the possibility that hemocyanin from other species may not possess this property especially in view of the differences in chemical behavior. Unfortunately for the purpose in mind neither octopus nor limulus blood was available and so no biological comparison could be made. As a source of hemocyanin use was made of the abalone (*Haliotis*) previously also used as a source for taurin (15), and the fluid was obtained in the manner recently described by Myers (16).

The opaque fluid was saturated with oxygen, filtered and an equal volume of saturated ammonium sulphate was slowly added, the fluid being constantly shaken to prevent local zones of high ammonium sulphate concentration. The blue precipitate was filtered off on hardened filter paper, then dissolved in a large volume of distilled water, and saturated ammonium sulphate again added to make a concentration of 4 cc. to each 10 cc. of solution. The precipitate was again filtered off, dissolved as before and reprecipitated by addition of ammonium sulphate to a concentration of 4.3 cc. per 10 cc. of fluid. The precipitate was filtered, redissolved, centrifuged to remove a trace of insoluble matter, and dialyzed first against running and then distilled water, toluol being used as a preservative. The salt-free solution contained a small amount of a white precipitate, probably hemocyanin from which the copper had been split. This was removed by centrifuging. The hemocyanin solution was concentrated by blowing warm dry air over the surface, made isotonic by addition of sodium chloride and for the purpose of injection preserved by the addition of phenol to a concentration of 0.25 per cent. The portion used for the final tests was preserved at a low temperature without addition of preservative.

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[2] These experiments came to my notice after the present work had been completed.

It will be noted that with respect to its behavior towards ammonium sulphate this preparation of hemocyanin was the same as that obtained by Alsberg from *Limulus polyphemus*. It might also be of interest in passing to mention that in comparison with the hemocyanin content of the fluid, the amounts of albumin and globulin were very small and it is very possible part of these consisted of hemocyanin.

Four normal rabbits were immunized by giving each 8 injections in doses of 80 mgm., a period of five days elapsing between the fourth and fifth injections. Ten days later the animals were bled, the sera inactivated and used for the subsequent tests. Fixation experiments using the well-known hemolytic system in the manner described in previous work with pure proteins (17), gave positive results, the limits of fixation, in terms of serum dilution, being as follows: Rabbit no. 25, 0.1 cc. of 1:250; rabbit no. 27, 0.1 cc. of 1:10; rabbit no. 29, 0.2 cc. of 1:250; rabbit no. 23, 0.4 cc. of 1:50. The usual controls were run to eliminate the possibility of inhibition of hemolysis by factors other than antibody. Positive precipitin tests were likewise given by the above sera. Addition of hemocyanin solution to a suspension of sheep red cells results neither in agglutination nor hemolysis of the red cells.

Three normal guinea-pigs were sensitized by giving each 8 mgm. subcutaneously and five weeks later were reinjected with results as follows: No. 1, 110 mgm. intravenously, death resulted in about ten minutes, symptoms typical of anaphylaxis; no. 2, 100 mgm. intraperitoneally, animal became very sick, temperature dropped 5°C., death resulted six hours after injection; no. 3, 100 mgm. intraperitoneally, slight drop in temperature, no visible symptoms noted.

Hemocyanin, like hemoglobin, is non-toxic. Two guinea pigs were given respectively 75 and 90 mgm. of hemocyanin solution intracardially and a third animal received 105 mgm. intraperitoneally. Visible symptoms were not shown and the temperature variation was within the normal variability.

These experiments furnish direct evidence that hemocyanin is antigenic and confirm the previous work of von Dungern and Cohnheim. Its chemical makeup must be very different from hemoglobin since the latter is non-antigenic.

SUMMARY

1. Hemocyanin prepared from the circulating fluid of the abalone (*Haliotis*) was precipitated by ammonium sulphate within the limits found by Alsberg and Clark from *Limulus polyphemus*. It appears to be a globulin.

2. The sera of rabbits immunized with this preparation of hemocyanin gave positive fixation and precipitin tests. It was toxic for guinea-pigs previously sensitized to this substance but not toxic for normal animals.

3. These experiments support the chemical viewpoint that the chemical makeup of hemocyanin is very different from hemoglobin since the latter is non-antigenic.

REFERENCES

(1) Kossel, A., and Kutscher, F.: Z. physiol. Chem., 1900–1901, **6**, 31, pp. 165–214, have shown that considerable differences exist in the amino acid content of salmin, clupein, cyclopterin, and sturin. Nevertheless these substances give the general reactions characteristic of the protamines. The differences in composition of the proteins of horse and ox serum noted by Hartley, P., Biochem. J., 1914, **8**, pp. 541–552, are not striking, and Gortner, R. A., and Wuertz, A. J., J. Amer. Chem. Soc., 1917, **39**, pp. 2239–2242, find that within the experimental error the composition of fibrin obtained from various animals is the same.

(2) Henze, M.: Z. f. physiol. Chem., 1901, **33**, pp. 370–384.
Henze, M.: Z. f. physiol. Chem., 1904, **43**, pp. 290–298.

(3) Alsberg, C. L., and Clark, E. D.: J. Biol. Chem., 1910, **8**, pp. 1–8.
Alsberg, C. L.: J. Biol. Chem., 1914, **19**, 77–82.

(4) Alsberg, C. L., and Clark, W. M.: J. Biol. Chem., 1914, **19**, 503–510.

(5) Dhéré, C.: J. Phyiol. et Path. gen., 1915, **16**, 985–997.
Dhéré, C.: J. Physiol. et Path. gen. 1919, **18**, 222–243.

(6) Wells, H. G., and Osborne, T. B.: J. Infect. Dis., 1911, **8**, 66–124.

(7) Van Slyke, D. D.: J. Biol. Chem., 1911–12, **10**, 15–55.

(8) Gay, F. P., and Robertson, T. B.: J. Exp. Med., 1913, **17**, 535-541.
Schmidt, C. L. A.: Univ. Cal. Pub. Path., 1916, **2**, 157-204.
Browning, C. H., and Wilson, G. H.: J. Path. & Bact., 1909, **14**, 174-183.
(9) Ford, W. W., and Halsey, J. T.: J. Med. Res., 1904, **11**, 403-425.
Levene, P. A.: J. Med. Res., 1904, **12**, 191-194.
Schmidt, C. L. A., and Bennett, C. B.: J. Infect. Dis., 1919, **25**, 207-212.
Bradley, H. C., and Sansum, W. D.: J. Biol. Chem., 1914, **18**, 497-506.
(10) Schittenhelm, A., and Weichardt, W.: Z. Immfrsch. & exp. Therap. Orig., 1912, **14**, 609-636.
(11) Wells, H. G.: J. Amer. Med. Assoc., 1908, **50**, 527-528.
(12) Nuttall, G. H. F.: Blood Immunity and Blood Relationship, Cambridge, 1904, p. 358.
(13) von Dungern, E.: Centralbl. Bakt. Ref., 1904-1905, **35**, 253.
(14) von Dungern, E., and Cohnheim, O.: Die Antikörper, Jena, 1903.
(15) Schmidt, C. L. A., and Watson, T.: J. Biol. Chem., 1918, **33**, 499-500.
(16) Myers, R. G.: J. Biol. Chem., 1920, **41**, 119-135.
(17) Schmidt, C. L. A.: Univ. of Cal. Pub. Path., 1916, **2**, 157-204.

ON THE NATURE OF BACTERIAL TOXAEMIA[1]

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I

On an occasion like this, when opportunity arises for students of immunity to meet for the discussion of their mutual problems, it seems fitting that, at least once during the meetings, the deliberations should take the form of a reëxamination of some of the fundamental premises on which our science is based. It is the business of most of us here to cast about continually for new leads, to leave the main highways of established fact and to follow more faintly marked paths to their endings. True progress can be made, and time and effort justified, only if we are sure, at each stage of our investigations that, so far, we have travelled straight. I assume that there are many of us here who have wasted effort on numerous occasions in trying to build at the top when the base needed support; and we all learn to recognize that endless repetition of experimental facts already well established, by slightly different methods and new manipulations, will bear dry fruit for science.

The astonishing progress which was made in the field of immunity during the last twenty or thirty years, was, in the main, based upon the careful and detailed "following through" of fundamental observations made by Pasteur, Behring, Richet, Metchnikoff, Bordet, Ehrlich, Theobald Smith and a few others. The application of these principles to the wealth of material which was made available by the rapid succession of etiological discoveries has kept students of this subject joyfully busy for two scientific generations. More recently, too, the possibilities of our field have been particularly enriched by the introduction of chemical and physical methods into the study of antigen-antibody reactions and by a development of the remarkable group of phenomena which we classify together as anaphylaxis. It is unnecessary to dwell upon the important yield of diagnostic, therapeutic, and prophylactic discoveries which has been contributed to medicine and biology as a result of these investigations. Indeed, these things constitute our entire scientific capital, and it is far from our intentions to minimize their great and fundamental importance. But it will be profitable, I think, at this stage of our development to sweep our eyes across the achievements of the past and to recognize that after all, this great edifice of facts is built upon the foundation of a few great principles, and that we are as ignorant as we were twenty years ago, and perhaps a little more confused, regarding some of the most important phenomena that accompany infection.

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In the diseases which are caused by bacteria that secrete true antigenic toxins, diphtheria, tetanus, and a few others, we have arrived at a fairly clear understanding of the mechanism by which the animal body is injured and of that by which it is protected.

In practically all of the remaining infections, however, there is uncertainty concerning many of the fundamental factors of attack and of defense. And in order to progress, we must first systematize out ignorance so that new work may not "take off" from premises of vague opinion or uncritically accepted assertion.

As regards the mechanism of injury, the problem about which there is most confusion is that concerning the nature of toxaemia. In practically all infectious diseases there are manifestations of local and general disturbances which cannot be attributed to mechanical injury by the growing bacteria. These take the form of fluctuations of temperature; of alterations, both quantitative and qualitative, of the leucocytes; of symptoms referable to the nervous system, such as depression, muscular and somatic pains, headache, nausea, weakness; and finally of local changes such as the inflammatory reactions in neighboring tissues, lymphatics and joints. In the last named instances it is of course debatable whether we are dealing with toxic disturbances rather than with local extensions of bacterial growth. But the toxic nature of the lymphangitis and oedema surrounding pyogenic foci or cellulitis is rendered likely by the speed with which these conditions subside upon drainage after incision, the proximal disappearance of the red lines marking lymph channels after tourniquet application, and such observations as that recently made by Auchincloss, in the case of a severe haemolytic streptococcus infection, in which he incised an intensely oedoematous area on the dorsum of the hand above the lesion, the copious fluid from which proved sterile in blood plate cultures.

It is hardly necessary to discuss these points at greater length, for it is obvious to all who have studied infections that the element of toxaemia, that is, the occurrence of disturbances remote from the actual seat of bacterial growth, is apparent in practically all diseases of bacterial origin, and there is a striking similarity in the nature of these manifestations, whatever the variety of infection. In such cases as typhoid fever, cholera, influenza, etc., the toxaemia is severe, while in surgical infections with pyogenic cocci it is relatively feeble; or, again, it is almost entirely absent, as in anthrax where the infected animal remains in astonishingly unchanged general condition until the later prelethal stages, when the body is flooded with bacteria. Nevertheless, except in a few instances like the last named, the toxic element is hardly ever entirely absent. Now when all is said and done, we know very little about the nature of the poisons which give rise to these disturbances, and about the mechanism by which they injure the body cells.

As regards defense reactions against infections in which true exotoxins play no rôle, we are a little better off in that we are in possession of accurate analyses of the serum antibodies and of their reactions with the bacterial antigens. But this after all is only a small part of the story. The reasons for the limited success of passive immunization with antibacterial sera; the nature of the participation of fixed tissue cells, both in temporary and permanently acquired resistance; these are only a few of the problems that must be solved before we can possess a clear understanding of the pathology of infection.

The obvious direction which empirical investigation has taken toward the solution of these problems has been the attempt to protect passively by the administration of sera with higher and higher antibody concentrations, in the hope that eventually this might change failure to success by quantitative improvement alone. To some extent it has seemed that this might actually solve the problems, inasmuch as the discovery of antigenic subgroups of organisms like the pneumococcus, the meningococcus, and the streptococcus indicated that early failures might have been due to lack of specificity on the part of the sera. But though this recognition has led to partial success in such conditions as type I pneumonia, yet in principle the difficulties have remained unchanged. To take the last named disease as an example, while a potent specific serum seems to possess distinct value in the treatment of type I pneumonia, yet the painstaking investigations of Cole and his associates have failed to obtain success in type II pneumococcus infections, although the organism is in most other respects entirely analogous to type I.

To be sure we are in possession of many important empirical observations, but to a large extent these are uncorrelated, and in some of the most important phases of immunity we are dependent upon trelisses of theory so flimsy that few of us have had the courage to climb them. At the bottom of most of our difficulties after all is the unclearness concerning the exact mechanism by which most of the pathogenic bacteria cause injury to the animal body. If we could thoroughly understand this, we might reason more clearly and plan our experimental projects more purposefully and in a less groping manner.

To some extent we have hitherto shirked this issue by assuming and teaching the existence of endotoxins in the original sense of Pfeiffer, although much has appeared in the literature which would indicate that the toxic constituents of the cell body do not tell the whole story. To formulate a clear judgment of this basic problem and to plan for further progress with a clear mind, it will be necessary to analyse critically the work that has been done in attempted explanations of bacterial toxaemia.

II

It is not necessary to recapitulate in detail the views of Pfeiffer It will be useful for the purposes of our subsequent discussion, however, to refresh our memories on certain phases of his experimental work which are often neglected when the existence of endotoxins is discussed.

In addition to showing that dead cholera spirilla, extracts of the spirilla and filtrates of old cultures, were more toxic than fresh filtrates, Pfeiffer obtained results which indicated that, by gradual reduction of the intraperitoneal dose we could kill guinea pigs with practically no living spirilla in the peritoneum at death. He also found that he could bring about rapid death in some of his animals by following the administration of considerable amounts of organisms with an injection of potent antiserum, a procedure aimed at the rapid dissolution of bacterial cells. These facts Pfeiffer interpreted as indicating that in cholera the injury to the body was brought about by the absorption of poisons liberated from the bacterial cells upon dissolution, and that this disintegration took place under the influence of the bacteriolytic serum constituents. This, in brief, extended to other bacteria, is the essence of the endotoxin theory.

From the beginning there was justifiable doubt regarding the specificity of these toxic substances. Differences in the symptoms induced by the poisons derived from different bacteria may be justly regarded as largely dependent upon quantitative variations. With the exception of the late paralytic effects observed with certain strains of dysentery and the frequent intestinal symptoms occurring in rabbits on inoculation with dysentery, typhoid paratyphoid, or colon extracts, there is no evidence of specificity, and even in these examples the specificity of such lesions requires more proof. At any rate, there is little evidence of tissue selection by the poisons themselves which can be regarded as in any way analogous to that exhibited by the true exotoxins of tetanus, diphtheria, and botulismus, and such selection as is evident in the human disease can be referred to selective distribution of the growing organisms.

Characteristic from the immunological point of view is the fact that the endotoxins are not generally conceived as possessing antitoxin inciting properties. The dissolved cell protoplasm gives rise to agglutinating, precipitating, etc., antibodies, and if any poison neutralizing properties are at all acquired by the serum of immunized animals, this never exceeds neutralizing power for a very limited number of lethal units; at any rate the law of multiples cannot be applied.

The first serious experimental objections to Pfeiffer's conception were those which regarded the poisonous properties of the bacterial cell not as the effects of preformed liberated poisons, but rather as the results of the proteolytic cleavage of the bacterial cell plasma. This point of view derived its intellectual stimulus from the work of Victor C. Vaughan upon protein split products. His observations on protein fever and the subsequent work of Ulrich Friedemann, who succeeded in rendering serum toxic in the course of specific hemolysis, gave a practical direction to this line of thought. Although Vaughan advanced the first experimental proof of toxic split products and their bearing upon the field of immunity, neither he nor Friedemann carried their theories beyond the realm of experimentation into that of fancy, a conservatism of which Friedberger, Embleton and Thiele and their associates cannot be accused. The conclusions of these workers have had much influence upon modern conceptions of toxaemia and must, therefore, be briefly discussed.

Friedberger produced his so-called "anaphylatoxins" from practically all varieties of bacteria, first by digesting them with sensitizer and alexin, then by treating them with normal guinea-pig serum alone. It is a very significant fact that he obtained them not only in the test tube, but on certain occasions within the guinea-pig peritoneum, a point significant in connection with Pfeiffer's earlier work. Friedberger and his collaborators regarded these toxic substances as split products of the bacterial protein which were formed whenever bacteria and active serum constituents were brought together under conditions of proper quantitative adjustment.

The opinion of Friedberger may be summarized as follows: Endotoxins in the original sense probably do not exist. When bacteria enter the body they are subjected to proteolytic action in which specific sensitizers and alexin play a part, the sensitizers present in normal serum of most animals and man being sufficient to initiate the process. In the course of cleavage a toxic protein split-product is formed, analogous to that obtained by Vaughan with chemical methods. This toxic substance is produced only during the early stages of the cleavage, but becomes non-toxic as proteolysis continues. All bacteria furnish the matrix for such a poison, and there is nothing specific about the process except the manner of its production by homologous antibodies. Whenever antibody and its antigen meet, the poison is produced; but in the animal body under conditions of infection this never occurs with sufficient speed nor in sufficient quantity to lead to acute death.

However, the symptoms caused by the repeated or continuous production of small amounts of this poison, constitute the basic manifestations of every infection; namely, local inflammation, fever, and injury to the central nervous system; and this is the explanation of systemic reactions occurring in the infected body, reactions which differ from one another not because of any specific differences in the nature of the poisons, but because of the varying abilities of the bacteria to invade, their accumulation and distribution, the degree of specific antibody formation, and the consequent speed of cleavage. The toxicity of these poisons is so great that minute amounts may give rise to symptoms. But when a relatively high concentration of antibodies occurs, as in recovery or immunization, the poisons are further broken down so rapidly that intoxication is prevented. In actual disease the accumulation of bacterial protein and the formation of antibodies are gradual processes during which the formation of "anaphylatoxins" and their further cleavage into non-toxic split products takes place constantly, and the balance between these two processes determines the intensity of the toxaemia.

Thiele and Embleton, though approaching the subject from a slightly different point of view, have gone even farther than Friedberger and his associates. They, too, believe that the so-called endotoxic action of bacteria is referable to products resulting from the cleavage of bacterial protoplasm by antibodies. They develop a theory of pathogenicity from this basic idea, the substance of which is that nonpathogenic bacteria are those against which antibody activities are either "so low" that toxic substances are liberated only in extremely small amounts; or "so high" that the further degradation of the protoplasm takes place with a rapidity that precluded the accumulation of the intermediate toxic substances. Between the two are the true pathogens.

Thiele and Embleton attempted to demonstrate the actual formation of these toxic substances in the bodies of infected animals by administering massive doses of bacteria to rabbits and guinea-pigs, bleeding them twenty-four to thirty-six hours later, and intravenously injecting the defibrinated or citrated blood, or even peritoneal exudate, into normal pigs. From the fact that these animals often died in acute convulsions they drew the conclusion that they had actually transferred poisons of bacterial origin.

Friedberger and Thiele and Embleton's work resulted in the construction of a very attractive and simple scheme for the explanation of toxaemia, virulence and the course of bacterial infections in general. Though they based their work upon the fundamental ideas advanced by Vaughan, they far outstrip him in comprehensiveness of the conclusions drawn. Vaughan, in summarizing the subject in 1913, mades the following statement:

"During the active progress of an infectious disease, the body cells supply the ferment, the infecting organism constitutes the substrate, the process is essentially destructive, the protein poison is set free, the symptoms of disease appear and life is placed in jeopardy."

It will be noted that Vaughan does not take for granted the formation of the poisons under the influence of the sensitizer-alexin mechanism in the circulation; and does not assume that the entire process necessarily takes place in the circulation.

The first important experimental objection brought forward against Friedberger's views was the observation of Keysser and Wassermann who showed that "anaphylatoxin" could be produced in guinea-pig serum by the use of kaolin and barium sulphate, without the presence of bacterial protein. Bordet showed that the same thing could be accomplished by incubating fresh guinea-pig serum for two hours with a small amount of weak agar solution. The same thing was done by Nathan and by Tschernoroutsky with amounts of agar as small as 0.01 mgm. per cubic centimeter of guinea-pig serum. Jobling and Peterson rendered serum toxic by treating it with chloroform. And finally Novy and De Kruiff in a very thorough and painstaking study confirmed these results in various ways, showing in addition that the important factor in the agar experiments is the physical state of the agar, and that ferment action is probably not at all involved. They found that inert sol-gel solutions injected intraveously into guinea-pigs, rats and rabbits gave rise to shock and other symptoms indistinguishable from anaphylatoxin effects; that the transfusion of blood from animals so treated occasionally showed toxic effects in the recipient guinea-pigs. Meanwhile, Köhler, Moldovan, Doerr, and others found that it is not even necessary to introduce foreign substances like kaolin or agar into the experiment. If blood is taken from animals in some manner by which clotting is delayed or prevented, as for instance by receiving it into paraffined vessels or by defibrinating or citrating, it may become toxic automatically, and cause death upon reinjection into animals of the same species or even into the same animal from which it was taken. In experiments done by Slatineau and Ciuca, guinea-pig serum reinjected into guinea-pigs within forty-five minutes, in large amounts, was shown to be occasionally toxic. De Kruiff especially confirmed and extended these observations, first by showing that rabbit blood can be rendered toxic and even fatal to guinea-pigs and white rats if transfused in the preclot period or after defibrination. Guinea-pig blood transferred unchanged within three minutes, or defibrinated or, on occasion, even the serum of such blood obtained by rapid clotting and centrifugation was often fatal to animals of the same species. We may summarize his more significant conclusions as follows: The spontaneous toxicity of normal blood develops in a similar manner as does the toxicity of blood treated with agar, peptone, etc. Poison production and fibrin formation go hand in hand and occur in the preclot stage.

The work of the writers mentioned, as well as much similar investigation of others renders untenable the theories of infection, virulence and bacterial toxaemia advanced by Friedberger and by Embleton and Thiele. The "anaphylatoxins" of Friedberger are quite surely not derived from the matrix of the bacterial cell plasma. Attempts to show bacterial poisons in the circulations of the infected animals by transfusion into other animals are not valid since the sera or the blood of normal animals may prove toxic if transferred in the same way. Moreover, it has never been shown and it is extremely unlikely that amboceptor or sensitizer and complement induce any sort of proteolytic process.

It should be distinctly understood, however, that these considerations do not eliminate the possibility of reactions between the bacterial antigen and the body cells and their ferments, as suggested by Vaughan in his book. Nor do they exclude the possibility that an accumulation of bacteria in the body, or their introduction into the blood from lesions might not cause injury in exactly the same way as does the injection of agar, peptone, bacterial protein, etc., in the anaphylatoxin experiment in animals. The mechanism, however, would be a similar one; that is, not a poisoning with a bacterial protein or its derivatives, but an injury due to a chemical or perhaps purely physical change incited in the blood, in which the poison is derived from the blood constituents themselves, and the bacteria play a rôle which De Kruiff has spoken of as analogous to a catalyst, but are otherwise inert.[2]

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[2] Incidentally, the work of Moldovan, Mita and Ito, Novy and De Kruiff, and others throws much interesting light upon the toxic effects which have been observed after the intravenous injection into human beings of such substances as bacterial vaccines, the much heralded phylacogens, and occasional similar observations with gum acacia solutions. They also promise to elucidate some of the hitherto obscure toxic effects of blood transfusion.

III

Let us now turn our attention to another line of investigation also dealing with the problem of bacterial toxaemia, but carried on by methods more directly aimed at the discovery of possible exotoxic antigenic substances from bacterial cultures. When we survey the literature which has accumulated upon this phase of the problem, we are struck by the fact that there is hardly a mircoörganism in the realm of pathogenic bacteria for which the claim of the existence of an antigenic toxin has not been made, at one time or another, and curiously enough, though none of these numerous claims have been generally accepted, and certainly none of them have been utilized to any extent in practical therapeutics, many of them seem based upon apparently valid experimental data, and in some cases such claims have been confirmed at intervals of a great many years by consecutive workers. The relatively slight impression which much of this work has made upon the development of immunity, is perhaps due to the fact that the antitoxic potency of the sera produced by immunization with such poisons has never been very high, and the results of clinical application have never been sufficiently impressive to carry conviction.

We cannot possibly undertake here to review the entire literature on this subject which has accumulated within the last twenty years, nor would it help us much in our analysis to do so. Much of it consists of repetition and confirmation of early results and many of the observations are so incomplete that it is difficult to evaluate them. However, it seems unquestionable that a large number of observers have actually obtained toxic substances from filtrates of extracts of many of the bacteria of which we are speaking, under circumstances which make it doubtful whether they were dealing with true cell proteins, that is endotoxins in the sense of Pfeiffer, or with bacterial products elaborated by the living bacteria and excreted into the culture fluids. Some of these observers have also presented reasonable evidence that the sera of animals immunized with their poisons have possessed true neutralizing properties.

Before we proceed with the analysis of such work, however, it seems to us worth while to consider briefly some of the underlying principles which should be kept in mind whenever a solution of the toxaemia problems by direct search for a poison is undertaken.

Whenever workers have turned their attention to investigations of this sort they have been to a certain extent under the spell of the analogy to the classic poisons of diphtheria, tetanus, etc. This is worth considering since it is important in clarifying our conceptions regarding these researches. The essential feature of poisons of this nature is not their potency, but rather whether or not they are bacterial secretions which are yielded as results of the metabolism of the living microörganisms, and whether they are truly antigenic. In the case of diphtheria, and in that of tetanus as well, we are dealing with bacteria that are essentially more saprophytic than parasitic organisms by which the living tissues are actually invaded, either not at all, or to a slight extent only. It is clear, therefore, that unless these bacteria which can multiply in the body locally only, produced very powerful poisons, they would not be pathogenic at all, and would not be brought to our attention in connection with human disease.

It is indeed quite conceivable that there may be a great many bacteria in nature that are as little invasive as these, and may produce true toxins of a weaker potency; these would never functionate as pathogens, simply because the weakness of their poisons and their lack of invasive power, taken together, render them entirely incapable of establishing a foothold in or upon the living body; or should this happen, of producing a sufficiently powerful poison, to incite symptoms. It is conversely not only conceivable, but rather likely that bacteria which possess that property which we describe as virulence or invasiveness, do not need to produce poisons of the degree of potency possessed by tetanus and diphtheria in order to cause symptoms of toxaemia in the invaded body. Small amounts of poisons of moderate potency would serve cumulatively to intoxicate the animal body if the bacteria can actively grow within it and it is, therefore, not essential to find poisons in broth or other cultures of such bacteria of anything approaching the potency of the classic ones mentioned in order to explain the toxaemias which accompany infection with these organisms.

It must be remembered, too, in analysing this literature and in carrying on further research, that it is by no means out of question that soluble poisons may be produced by bacteria in the course of their growth which are neither extract products nor antigenic. They may not even be specific. If we insist upon the conformity of many poisons with these criteria before we include them in our considerations of the causations of bacterial toxaemia, we may be missing the truth. For just as the peptic, tryptic, and other intestinal enzymes which perform similar tasks in many different animals, are functionally alike, so the products of many bacteria may be similar to each other, whatever the species. And, indeed, such similarity of product seems particularly likely in a group of living things which are so essentially alike in basic metabolism and morphology as the bacteria. How much this last possibility should be considered will become clearer as we go on. At the present moment we wish simply to insist that in seeking for the cause of bacterial toxaemia we must emancipate ourselves from the suppositions that all the poisons we observe must fit into the mold of earlier investigations, if they are to be at all included in our principles of reasoning.

For the purposes of our presentation today, we have tabulated these researches since it would take a needless amount of time and space to analyze each one separately.

So many important bacteriological principles are involved in the researches just tabulated that no superficial general analysis can do them justice. A considerable degree of clearness, however, can be obtained by comparing the results arrived at in regard to the more basic facts.

Two main principles of poison production have been employed. One of these has been aimed at the extraction of the substances of the cell bodies either by grinding and extracting growths on solid media or by filtration of old liquid cultures. These poisons, quite obviously, are analogous to the endotoxins of Pfeiffer and, although the literature is not entirely consistent, there seems to be a more or less general agreement that this class of poisons has exhibited little specificity, that they have been toxic for both rabbits and guinea-pigs, and have been found relatively stable in their resistance to heat and deterioration on standing.

Other methods have aimed to produce poisons with as little extraction of the bacterial cell body as possible, either by filtration of younger liquid cultures or by the rapid washing up of young agar growths with salt solution, and immediate filtration of these substances.

The manifold investigations in which the nature of the experiments makes it quite impossible to determine to what degree extraction products may have been admixed with the products of metabolism of the living microörganisms, is the feature which has led to so many divergent results and renders general analysis so difficult.

However, in those cases in which the manner of poison production was such that it is reasonable to believe that cell body extracts played a minor part only in the final toxicity of the filtrates, there has been a certain amount of regularity of behavior which serves to characterise such poisons as different from the endotoxic products obtained with the dead bacteria or cell extracts of the same species. Thus, while Besredka's typhoid cell extracts are stated as possessing considerable stability, Kraus's nine day filtrates deteriorated rapidly, Aronson's ten day filtrates were destroyed at 80°C. and deteriorated in a short time, and Yamanouchi's seven day filtrates were partly destroyed at 60°C. in thirty minutes.

Similarly, in dysentery, cell body poisons have generally been found to resist heating up to 90°C. and above. But Kraus and Doerr's poisons from agar washings were destroyed at 80°C. in a few minutes; Todd's 10 day filtrates were unstable on standing, and were destroyed by 70° to 80°C. in an hour; and Olitsky and Kligler's "exotoxins" were destroyed by 75°C. in one hour, while 90°C. for the same time was required for the destruction of their cellular poisons.

With streptococci, endotoxic cell extracts have been satisfactorily demonstrated, and the toxic substances occasionally observed in broth filtrates have usually been unstable.

The *B. influenzae* filtrates of Mrs. Parker in which the time of cultivation varied anywhere from six to eighteen hours, have been found to lose potency at 70°C., as have similar filtrates produced with other organisms in our own laboratory.

Again, these supposedly exotoxic substances have in many cases failed to show toxicity for guinea-pigs. Kraus and Doerr's dysentery agar washings, with which they produced neutralizing sera were entirely innocuous for guinea-pigs and in Kraus and Stenitzer's work on typhoid, potency for guinea-pigs was very weak. This is likewise true of Arima's typhoid exotoxins, and has been the experience generally with streptococcus poisons. The same thing is true of Mrs. Parker's poisons of *B. influenzae*, and all of the toxic substances produced by filtrates of early cultures of various organisms which we will described below.

It seems more than probable, therefore, that in broth filtrates of relatively young cultures as well as in the filtrates of agar washings, we are dealing with poisons distinct from the endotoxic substances, and such supposedly exotoxic products have shown remarkably points of similarity in regard to instability, and relatively low toxicity for guinea-pigs. Furthermore, it is significant in this connection that different organisms have occasionally shown analogous differences in the symptom complexes produced by the stable and the unstable poisons. With the Shiga dysentery bacilli, Kraus, Bessau, Dopter and more recently Olitsky and Kligler have shown that filtrates of the younger cultures of this organism produced paralytic symptoms in rabbits, while the

Typhoid

WORKERS AND DATES	METHOD OF PRODUCTION	TOXICITY FOR		STABILITY	ANTITOXIN PRODUCTION	REMARKS
		Rabbits	Guinea-pigs			
Besredka, Ann. de l'Inst. Past., 1905, vol. 19	Aga culture, dry bacilli. Killed at 60°C. ground with salt. 0.15 gram and 2 cc. of salt solution and 8 cc. serum together 2 hours, then centrifuged	(?) Incubation time (?)	1.5 cc. kills guinea-pig of 300 grams	Resists 57°C. for 15 hours	Yes. Horse immune serum 0.15 cc. protects against 15 fatal doses	Could he have been dealing with anaphylatoxin a similar toxification of the horse serum by the bacteria à la Novy and DeKruiff
Arima, C. f. Bakt., 1912, vol. 63.	*Exotoxin.* Washing up of bacilli in saline and centrifugating without interval	++++, for mice, guinea-pigs, rabbits, goats, but especially for goats, and rabbits		Nothing said (?)	Exotoxin gave paralysis in rabbits and diarrhea.	
	Endotoxin. Sediment of above, ground up	The same		(?)	Characteristic intestinal lesions	
Conradi, Deut. med. Wochenschr., 1906, vol. 32	20 hour typhoid culture, autolyzed in salt solution 48 hours, filtered and evaporated at 35° to one-tenth its volume		0.2 cc. killed 8 guinea-pigs of 300 grams	(?)	(?)	

Kraus and Stenitzer, Ztschr. f. Imm., 1909, vol. 3 See also Kraus, Wien. Kl. Woch. (below)	(1) 9 day broth culture, filtered through paper (2) 24 hour agar culture washed off with water, salt or weak soda. Allowed to stand 12 to 24 hours. Filtered through paper	2 cc. fatal ++++	Weak Less	Unstable, but no specific data are given	Immunising with the agar culture extracts sera were obtained that neutralised 2 or 3 lethal doses	
Aronson, Berl. K. Woch., 1907, vol. 44, p. 572	Point of pellicle growth on broth; 8 to 10 days growth; filtrate	2 to 5 cc. kills rabbit of 1000 grams in 2 to 6 hours	Not used	Deteriorates rapidly; destroyed at 80°C.; does not diffuse.	Moderate; must be injected 24 hours before	
Yamanouchi, C. R. Soc. Biol., 1909, vol. 61, p. 1051	7 day filtrates on 5 per cent peptone; filter through Chamberland candles; becomes toxic on 3rd day	0.5 to 1 cc. kills 1 kilo rabbit in 2 to 6 hours; 12 hours at most	Not used	Destroyed partly at 60°C. in 30 minutes; altogether at 100°C.	Neutralized by anti-endotoxin of Besredka; best leave with serum in incubator for 30 minutes; separately injected there was no neutralization	Similar, but feebler poison produced by paratyphoid A and B. Gartner bacillus produced none, and para-poisons were not neutralized by Besredka's serum
MacFadyen and Rowland, C. f. B., 1901, vol. 30, and MacFadyen, C. f. B., 1906, vol. 41	Grinding in frozen condition and extraction with 0.1 per cent KOH	++++, and also for goats	Very slightly for guinea-pigs	Labile	Yes. 30 doses neutralised	

Typhoid—Continued

WORKERS AND DATES	METHOD OF PRODUCTION	TOXICITY FOR		STABILITY	ANTITOXIN PRODUCTION	REMARKS
		Rabbits	Guinea-pigs			
Meyer and Bergell, Berl. k. Woch., 1907, vol 44, p. 568	Agar cultures washed up in 20 cc. slightly alkaline distilled water; room temperature 48 hours; filtered through Chamberland filter	++++, 3 to 4 cc. death in 12 hours	(?)	+, Deteriorated on standing within 24 hours. No change in complement fixation nor in precipitation	Injected horse yielded serum which in small amounts neutralized 2 lethal doses	
Kraus, Wien. klin. Woch., 1907, vol. 20, p. 344	9 to 40 day filtrates	++++, incubation time short; speaks of death in 5 hours with 1 cc.		Very labile; deteriorated in ice chest in 1 week	He neutralized his poisons to degree of 1⅓ fatal doses with his own and with Besredka's serum	But gives no particulars as to details of technique of neutralization, whether injected together after incubation, etc.

Dysentery

Doerr; and Kraus and Doerr, Kraus and Levaditi Handbuch, vols. 1 and 2	Alkalinity necessary; aerobiosis; culture filtered after 2 to 3 weeks; best through paper after carbolizing	++++, rabbits most susceptible; monkeys ++; paralytic symptoms in rabbits	Guinea pigs only bacterial bodies are toxic. Broth filtrates not toxic for guinea-pigs; "vŏllig refraktăr"	Destroyed at 80°C. in a few minutes	Law of multiples established with the Vienna serum; a lethal dose was neutralized by 0.005 cc. of the serum; the serum could neutralize as high as 20 doses	

Kraus and Doerr, Ztschr. f. Hyg., 1906, vol. 55	Kraus, 20 hour agar culture washed up in saline and allowed to stand 1 hour at room temperature and filtered; also Massenkulturen einige Minuten schütteln und durch Reichelkerzen filtriert	Incubation time short after intravenous injection; authors speak of death in 6 hours; with our poisons this would mean symptoms within 30 to 90 minutes; very weak if subcutaneously given; innocuous when intestinally introduced			Noticeable that the sera first obtained in early bleeding neutralized only when mixed in vitro Even later they failed to neutralize if subcutaneously injected together. Only the strongest sera showed any effects after the rabbit had become sick	
Kraus and Doerr, loc. cit.	Two methods; filtration	3 weeks old cultures much more powerful than 9 days or younger cultures; differences between different strains; filtrate injections gave exactly the same symptoms as injections of killed organisms; but the filtrates acted more quickly. Paralysis would often appear with these poisons within a few hours; death in 24 to 36 hours. Death on subcutaneous and intraperitoneal injection, but large doses necessary. Paralytic symptoms typical. These poisons absolutely harmless for guinea-pigs			Antitoxin produced in goats and horses; serum only potent after prolonged immunization, a much weaker but also noticeable neutralization took place when the antitoxin and toxin were separately injected; but if the antitoxin was delayed by 5 minutes increased quantities of the serum were no longer curative	
	Agar cultures were washed up in salt solution, shaken for few minutes and filtered. Autolysis was excluded by this method. Similarly prepared cultures of the Flexner bacillus gave negative results (grown anywhere from 8 to 20 days or longer)					
Todd, Brit. Med. Journ., December, 1903, vol. 2, p. 1456	Alkaline peptone broth; 10 day growth filtered. Does not give length of "latent period"	Rabbit ++++, horse ++++; rabbit killed with 1 cc. intravenously	Mouse and monkey negative	Destroyed at 70° to 80°C. for 1 hour; nothing said about standing in incubator	Difficulty in immunizing horse or goats; one horse killed; another well after 4 months	Horse serum; 1 cc. protected against 80 lethal doses of poison; best results when toxin and antitoxin were allowed to stand for 1 hour in incubator. *When mixed and immediately injected the effects were less favorable*

Dysentery—*Continued*

WORKERS AND DATES	METHOD OF PRODUCTION	TOXICITY FOR		STABILITY	ANTITOXIN PRODUCTION	REMARKS
		Rabbits	Guinea-pigs			
Olitsky and Kligler, (Shiga), Journ. Exp. Med., 1920, vol. 31, p. 19	*Exotoxin.* 5 day growth in alkaline egg broth filtered; incubation few hours to 4 days.	++++, paralysis and severe nerve lesions		75°C. for 1 hour killed it; authors say nothing about deterioration on standing	Specific neutralization of 1000 lethal doses of poison	
	Endotoxin. organism grown on agar, washed off in salt solution, incubated 2 days and filtered. Incubation time only fact given 24 to 48 hours	++++, diarrhea, loss of weight, no paralysis		90°C. for 1 hour		
Pfeiffer and Ungermann, Cent. f. Bakt., Orig., 1909, vol. 50	Showed the Kraus antitoxic serum. Believe it to have true antitoxic action but only when injected with toxin. The toxin which kills guinea-pigs produces no antitoxin					

Bessau, C. f. Bakt., 1911, vol. 57, p. 27	Also finds two poisons, one which produces paralysis, the other marasmus; both included in the killed whole bacillus bodies	Rabbits, ++++ ++++			True antitoxin in the Kraus serum against paretic poisons	He differentiated the two poisons by the fact that the paretic action was neutralized by the Kraus serum, the other not. He may have been working without knowing it with the soluble substances obtained in agar washings in addition to the cell bodies, since he washed off his cultures with salt and heated only to 56°C.
			Cholera			
Brau and Denier, C. R. Soc. Biol., 1904, vol. 56, p. 433	Used medium of defibrinated horse blood and horse serum. Heated at 60°C. for 3 hours !!! 7 day filtrates used	Much less than for pigs but 0.5 to 1.5 intravenously kills them	0.5 cc., ++++, also dog and horse, ++++; mice negative	Very stable; destroyed by 120°C. for 20 minutes !!! toxicity for rabbits lost more rapidly than for guinea-pigs	Yes. Moderate degree of neutralization. Serum is antibacterial as well as antitoxic. Normal serum mixed with the poison in vitro neutralizes 1 to 2 lethal doses "antitoxic" serum	Mixed in vitro their horse immune serum (½ hour at 37 after mixing): 1/50 neutralizes 2 doses 1/20 neutralizes 3 doses 1 cc. neutralizes 4 doses 2 cc. neutralizes 6 doses Injected before poison it protects Simultaneously it is "moins favorable"
Brau and Denier, Ann. de l'Inst. Pasteur, 1906, vol. 20, p. 578	Toxicity begins on 4th day. Oxygenation helps. Toxicity diminishes if sp. passed the animals	"No incubation period," p. 584				

Cholera—Continued

WORKERS AND DATES	METHOD OF PRODUCTION	TOXICITY FOR		STABILITY	ANTITOXIN PRODUCTION	REMARKS
		Rabbits	Guinea-pigs			
Kraus, Kolle and Wassermann Handbuch, vol. 1, p. 180	Beef broth peptone; optimum in 5 days to 2 weeks; loss in filtration appears as early as 3 days	Less toxic than for guinea-pigs	1 to 3 cc. within from 5 to 24 hours	Deteriorates slowly; markedly diminished at 70°C. in 30 minutes	Neutralized to some degree. Serum must be injected within 10 minutes after toxin. Best mixed in vitro	
		Very short incubation time; 10 to 30 minutes with large doses; death in 5 hours				
MacFadyen, C. f. Bact., 1906, vol. 42, 365	Ground spirilla (exotoxin) in 0.1 per cent KOH as in his typhoid method				Serum of a goat neutralized the toxin as high as 10 lethal doses. Mixed and allowed to stand at 39°C. for an hour or longer	
See also earlier work of Roux and Salimbeni with Metchnikoff, Ann. de l'Inst. Past., 1896, vol. 10, p. 257						

Streptococcus

WORKERS AND DATES	METHOD OF PRODUCTION	Rabbits	Guinea-pigs	STABILITY	ANTITOXIN PRODUCTION	REMARKS
Marmorek, Ann. Past., 1902, vol. 16, p. 169	Complicated medium of glycocoll and leucin broth with guinea-pig leucocytes grown 8 days and filtered	++++, 0.25 to 0.5 kills rabbit weight (?)	(?) Not given	Destroyed at 70°C.	Claims yes, but claim is not well substantiated	

Braun, C. f. Bact., 1912, vol. 61, p. 383	10 hour filtrates	++++, irregular + in 24 hours or slow diarrhea, emaciation	No effect on guinea-pigs	Deterioration in incubator rapid		Braun thinks strains of great growth energy must be used. Maximum growth time best; before deterioration; absorption of poison by filter
Aronson, Berl. k. Woch., 1902, vol. 39	Chiefly negative except when 10 to 20 cc. were used. Sediments entirely negative even in large amounts. Local infiltration and occasional deaths with filtrates from 2 to 4 days old. Very slight toxicity in the streptococcus body. 70°C. diminished the toxicity of all the piosons					
Simons, C. f. Bact. 1904, vol. 35	No poisons in bodies of cocci.	No relationship between toxin and hemolysin. Toxin which killed rabbits produced in anaerobic cultures on aleuronat exudate and broth filtered after 2 to 9 days. Irregular results. Always emaciation and diarrhea. "Streptococci produce poisons only in contact with animal exudates, leucocytes or other harmful animal influences"				
Clark and Fenton, J. A. M. A., 1918, vol. 71, 1048	Cultivated in Locke's solution with defibrinated blood and 0.5 per cent glucose filtration in 48 hours	0.5 to 1 cc. per kilogram killed. Incubation time 14 to 24 hours	Not mentioned	Toxicity destroyed at 50°C. for 30 minutes; diminished in a few days in the ice-box	Resistance to 6 lethal doses by active immunization. Immunity against living cultures conferred. No passive immunization attempted	Hemoglobin absolutely necessary for toxin production. Doses dialyzed through celloidin

older extraction poisons or direct sediment extracts have failed to cause paralysis, but have given rise to intestinal symptoms and marasmus. And Arima's work with typhoid bacilli gave exactly analogous results with these organisms in that his so-called typhoid exotoxin gave him paralysis, while the cell body poisons produced intestinal symptoms and marasmus.

As regards the serological work done to prove the true toxin nature and the specificity of such poisons, there can be little doubt about the fact that a great many observers, especially those who have worked with typhoid, dysentery, and cholera poisons, have produced sera which neutralized the toxic substances to a limited extent. It is worth noting, however, that in a great many instances there has been a very marked difference in the results when the poison and the serum were injected separately, though simultaneously, or when the two have been mixed and kept in the incubator for from thirty minutes to one hour before injection. Very powerful neutralization has been observed only in the latter case, and since in most of the poisons the presence of true bacterial antigen cannot be excluded, it is by no means impossible that many of these neutralizations, at any rate, depended upon union of dissolved bacterial antigen and its antibody in the incubator before injection.

It is noticeable, too, that the sera produced with extracts of typhoid cell bodies by Besredka possessed neutralizing action for the poisons obtained by Yamanouchi in seven day filtrates and by Kraus in nine day filtrates, Also Bessau, using as poisons the killed bodies of the dysentery bacillus, found that Kraus' dysentery serum produced with filtrates and filtered agar washings neutralized the paralytic poisons of his endotoxins, but not the marasmic effects.[3]

IV

Our own work was undertaken because we felt that there was much unclearness regarding many of the claims that had been made by various investigators regarding the so-called exotoxins of bacteria, and we believed that no immediate progress of importance could result from further investigations of the endotoxin problem until the existence of true exotoxins and their properties were definitely settled. Also we were induced to take up this matter particularly because observations made by us with toxic filtrates of haemolytic streptococci indicated that there was a striking qualitative similarity between these and the *B. influenzae* filtrates produced in parallel experiments by Mrs. Parker. We therefore decided to undertake, with the assistance of Mrs. Parker and Miss Kuttner, a detailed study of poisons of the possible exotoxic variety with a considerable number of different bacteria.

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[3] For a possible explanation of this see remarks in tabulation under Bessau.

Throughout our work we have endeavored to avoid as far as possible any extensive extraction of the bacterial cell. For this reason we have worked chiefly with filtrates of very young cultures on liquid media and with filtered salt solution washings of young agar growths.

Our work is far from completion and is mentioned in this paper only because some of our preliminary results seem to us to have important direct bearing on the problems discussed above. In addition to streptococci and influenza bacilli we have, so far, employed especially the typhoid bacillus and have done a few isolated experiments of orientation with dysentery bacilli of the Shiga and Flexner groups, the bacillus prodigiosus, the colon bacillus, staphylococcus aureus and the meningococcus. The details of these experiments will be analyzed and presented at some furture time. For the present we wish to submit the following data.

Working with various strains of haemolytic streptococci we have found that culture filtrates, (better centrifugates, since the filter detracts from toxicity) can often be shown to possess toxicity for rabbits. The best results are obtained by cultivation under conditions of partial anaerobiosis (20 cm. of mercury) for twenty-two hours. The poisons appear to some extent on simple hormone broth and upon ascitic broth but they are most powerful when the streptococci are cultivated on horse chocolate broth. They are at best never very powerful, 3 to 5 cc. always make rabbits of 1200 to 1500 grams very sick, but the action is irregular in intensity. We have killed in anywhere from five hours to three or four days with these poisons but many rabbits eventually recover in spite of a degree of illness that would lead the inexperienced to expect certain death. The poisons are destroyed at 75°C. to 80°C. in thirty minutes, and they seem to deteriorate with considerable speed on standing, though the details of this are not yet worked out. In this, as in the sequence of symptoms these toxic substances bear great similarity to the influenza poisons found and reported by Mrs. Parker. The incubation time is short. Rabbits that eventually die get sick within forty-five minutes to an hour and one half. There is weakness, often diarrhea, respiratory difficulty and a curious watering of the eyes. In other respects the rabbits act much like animals injected with a toxic foreign serum. Weakness is progressive and, finally, after three, four or five hours or longer the animals lie on their sides unable to rise, in general muscular paresis, but without paralysis and die, usually without convulsions. Autopsy shows nothing but congestion of the abdominal viscera especially the mesentery and bowel, and sometimes a little exudation in the serous cavities. The poisons, like the influenza ones, have no action on guinea-pigs and little or none on mice. All necessary controls were of course done, and will be reported.

Typhoid filtrates have been prepared with various culture media, and most of the experiments have been done with cultures five and a half to six hours old. Such filtrates were always powerfully toxic for rabbits, with a potency midway between the more powerful influenza poisons and the weaker streptococcus ones. In all the other properties mentioned above they were qualitatively identical with the streptococcus and influenza poisons. In order to compare these substances with the cell extracts we have carried out several comparisons between the six hour filtrates and similarly prepared filtrates from six day to ten day typhoid cultures. On several occasions we have found that the 6 hour filtrates were as toxic for rabbits (in one case even more so) than the ten day ones, but while the former were innocuous for guinea-pigs in relatively high dose, the latter killed guinea-pigs acutely. These young typhoid filtrate poisons were also destroyed at 75°C. in half an hour, but their deterioration index has not yet been fully worked out. Other filtrate experiments are as yet so incomplete that they cannot properly be included.

The first source of error in such experiments that come to one's mind is, of course, the possibility of toxic substances of this kind having originated from changes wrought upon the culture medium by the growing bacteria, a thought which is particularly obvious in view of the striking similarity in heat resistance and mode of production, and the complete identity of physiological action of such poisons from different bacteria. How far this can be absolutely excluded, it is hard to say. We can rule out histamin (shown recently to be produced on seven and ten day colon cultures) by the inability of our toxic substances to act on the isolated guinea-pig uterus, by their innocuousness for guinea-pigs and by heat instability. The last point would tend to exclude also most of the substances belonging to the ptomain series, though about some of these we cannot find statements concerning this property based on experiment. Peptone effects we can exclude with considerable certainty, we think, since our original culture media in considerable amounts do not give the symptoms; because 20 cc. of a 5 per cent solution made with our peptone gave no immediate symptoms in a rabbit, killing only after six days with nothing resembling the symptoms we were obtaining with 3 to 5 cc. of our 1 per cent peptone broth filtrates; because guinea-pigs were unaffected and because heat at 75°C. destroyed the toxicity. Cholin derivatives have not yet been excluded and unknown possible toxic culture ingredients have not yet been fully searched for by us.

In order to get a more complete understanding of a few of these poisons, however, before we completed all the details concerning the broth filtrates, we began to work with agar cultures in which, as in the dysentery work of Kraus and the typhoid work of Arima, the bacteria were grown on agar surfaces, the growths washed off with sterile salt solution and filtered with as little interval as possible between removal and filtration and with care that no agar was scraped off, usually without any scraping of the agar whatever. By this method, astonishing as it was to us, we obtained powerfully toxic filtrates from influenza, typhoid, colon and prodigiosus cultures; less powerful ones from streptococcus growths and from growths of dysentery bacilli. In all cases the differences between these various toxic substances were purely those of potency. Qualitatively they were similar to each other and to the poisons obtained from broth filtrates, at least as far as their toxicity for rabbits, the resulting symptoms and autopsy findings and the incubation periods were concerned. Quantitative comparisons must be made in these experiments since the perhaps confusing impression given by the equal toxicity of substances obtained from the saprophytic organisms with those obtained from the pathogenic ones may lose some of its negative significance when we remember how incomparably more abundant were the six hour growths on agar of prodigiosus and bacillus coli. We have excluded the possible coöperation of an agar anaphylatoxin in these effects, we believe, a control which, by the way, was not made either by Kraus or Arima in their work.

So far, therefore, although we have made only a beginning in a rather ambitious program, we have found that many different bacteria will induce the formation of heat unstable toxic substances in young cultures. The formation of this substance is roughly proportionate to the growth energy. The toxic products are essentially similar in the symptoms they elicit in rabbits and they are similar in their harmlessness for guinea-pigs. They differ in some essential properties from the classical endotoxins of the same organisms, and though we cannot yet be absolutely sure of it, they seem distinct from most of the more usual toxic substances produced by the cleavage of culture ingredients. If the broth filtrate substances are identical with the poisons obtained from the agar washings, a fact that we deem more than likely at present, we can probably exclude the coöperation of culture substances definitely. We think that there is little doubt that the substance we have under observation is at any rate responsible for a good many vaguely comprehended results obtained by preceding investigators.

The essential questions of identity of these substances from different bacteria, their antigenic properties, etc., can be answered only by animal experiments. Such immunization and cross protection experiments are in progress, and they are rendered extremely difficult by the fact that repeated small doses often lead to marasmus, loss of hair and eventual death of animals so treated.

The final question as to whether these poisons play any rôle in the symptoms accompanying infection of the animal body will be more difficult of approach and will not be attacked until some of the more basic problems have been answered.

One noticeable feature that may have some bearing on these substances is the apparent aggressive action of our poisonous products. Occasionally rabbits that were injected with unquestionably sublethal doses of streptococci contained in supernatant fluid from centrifuged specimens have died in about two or three days; and with the poison of *B. influenzae* the small number of influenza organisms that occasionally slip through the filters have caused death in the rabbits with invasion of the tissues, although this organism in its ordinary relationship does not infect rabbits.

There remains one further possibility of injury to the body which has nothing to do with toxaemia in the sense of the production of free bacterial poisons. There is no conclusive evidence at the present time which would lead us to doubt that in chronic infection and in repeated infection with the same organism the body may become sensitized to the antigen of this organism. Studies on the typhoidin reaction, and to a less definite extent on the tuberculin reaction, would point in this direction. Investigations on the active and passive sensitization of guinea-pigs against typhoid protein which we published some years ago, tend to indicate that just before, or at the time when antibodies appear in the circulation, there have developed sessile antibodies of the same nature. The violent reaction of guinea-pig uteri existing at such times; the partial protection of the cells in such cases by increased concentrations of the circulating antibodies; these facts would, at least, suggest that such a mechanism can play a distinct rôle in cases of prolonged, chronic repeated infections. But whether or not such animal experiments can be translated into the conditions prevailing in the infected human body, must rest upon further study.

V

It is quite apparent from the preceding considerations that a simple answer for the complex problems of bacterial toxaemia is quite out of the question. Indeed, we have perhaps been illogical in hoping to explain by any simple mechanism a series of phenomena as intricate as these, in which there is a struggle between two rival metabolisms, mutual modification and injury. Yet, difficult as the problem may be, there is hope of ultimate clearness. In the attainment of this we can best assist by selecting from the work of the past that which may be accepted as permanent fact, that which may be set aside, and that which may, for purposes of experimentation, be regarded as rational possibility.

For the time being this may be done as follows:

1. The body substances of most Gram-negative bacteria are toxic for the ordinary laboratory animals. These toxic properties are common to many non-pathogenic, as well as pathogenic bacteria of this class. It is uncertain, but unlikely, that they are pharmacologically specific.

These substances can be obtained by a variety of extraction methods, as well as by prolonged cultivation in broth.

In a large majority of cases these substances have been found relatively resistant to heat, and do not deteriorate readily on standing.

They do not induce neutralizing antibodies of any marked degree of potency, but they do induce specific protein sensitizers by means of which partial specific neutralization of their effects may be accomplished.

These are the so-called endotoxins.

The mechanism of the action of these substances is somewhat uncertain. They certainly do not form the matrix of toxic split products produced in the circulation by the sensitizer-alexin complex as conceived by Friedberger, and others; but injury by their reaction with the fixed tissue cells as conceived by Vaughan cannot be excluded, and, indeed, there seems to be no experimental method of differentiating such reaction from the direct poisoning of tissue cells by preformed poisons of such cell substances.

Injury by their purely physical effects in the circulation as suggested, among others, by Novy and De Kruiff, is unlikely, but must be considered.

Similar endotoxic substances have not been consistently produced with Gram-positive bacteria.

2. Toxic substances which are probably not identical with the poisons of the bacterial cell body have been produced with many pathogenic and some non-pathogenic bacteria, both Gram-negative and Gram-positive, by the filtration of young cultures, and by filtering washings of agar growths.

No conclusive evidence has been brought so far to show that poisons produced by these two methods are not identical.

The poisons produced by these two methods seem to be exotoxic in the sense that they do not represent extraction products.

These substances are less heat stable than the endotoxins, usually being destroyed by 75° to 80°C. in thirty minutes.

They have usually possessed relatively low toxicity for guinea-pigs.

Poisons produced by these two methods in our own laboratory from a considerable number of bacteria have been found identical in regard to heat resistance, innocuousness for guinea-pigs, incubation time, physiological action, and autopsy findings in rabbits. This, to us, reopens the question of whether these poisons are any more specific than are the endotoxins described above.

This problem can be settled only by extensive experiments on specific immunization and active and passive cross immunization. So far, attempts to increase the resistance of rabbits against these poisons, as produced by us, have met with little success, since repeated injection led to emaciation, loss of hair, marasmus and death. (Recently, 10 out of 11 rabbits carefully treated with slightly increasing doses of young typhoid and streptococcus filtrates have died, and a horse, placed at our disposal for similar treatment with influenza filtrates made in our laboratory by Mrs. Parker, also died in the course of immunization.)

It is still necessary to identify the poisons we are working with, with similar exotoxic products of other investigators. To us this identity seems likely at the present time.

Specific antigenic effects of poisons of this class produced from a variety of bacteria have been claimed by a number of reliable investigators. Reviewing this part of the problem as a whole, it becomes apparent that nothing further can be said until more extensive immunization experiments have been completed.

When these preliminaries have been disposed of, we will be in a position to proceed to a study of the relative importance of these toxic bacterial products in the infectious diseases of man and animals, and only then will we be able to see our way clear to the practical problems of serum therapeutics.

REFERENCES

Pfeiffer: Jahresbericht f. Immunität., 1910, **6**, 13. (critical summary by Pfeiffer himself).

Vaughan, Victor C.: Protein split products in relation to immunity and disease. Lea and Febiger, New York, 1913.

Friedemann, Ulrich: Zeitschr. f. Immunität., orig., 1909, **2**, 591, ibid., 1909, **3**, 726; Jahresbericht f. Immunität., 1910, **6**, 31.

Friedberger and collaborators: Zeitschr. f. Immunität., orig., 1910, **6**, 179, 299; ibid., 1910, **7**, 94, 665, 748.

Thiele (Teale) and Embleton: Zeitschr. f. Immunität., orig., 1913, **19**, 643, 666.

Keysser and Wassermann: Zeitschr. f. Hyg., 1911, **68**, 535.

Bordet: Compt. Rend. de la Soc. de Biol., 1913, **74**, 225.

Nathan: Zeitschr. f. Immunitat., orig., 1913, **17**, 478.

Tchernoroutzky: Compt. Rend. de la Soc. de Biol., 1913, **74**, 1213.

Jobling and Peterson: Jour. of Exp. Med., 1914, **19**, 485.

Novy and De Kruif: Jour. of Inf. Dis., 1917, **20**, 499 et seq.

Kohler: Cited from Novy and De Kruif, loc. cit.

Moldovan: Deut. Med. Woch., 1910, **36**, 2422.

Doerr: Wien. Klin. Woch., 1912, **25**, 331, 339.

Slatineano and Ciuca: Compt. Rend de la Soc. de Biol., 1913, **74**, 631.

De Kruif: loc. cit. (Novy and De Kruif.)

Mita and Ito: Zeitschr. f. Immunität., orig., 1913, **17**, 586.

Besredka: Ann. de. l'Inst. Pasteur, 1905, **19**, 477.

Arima: Central. f. Bakt., orig., 1912, **63**, 424.

Conradi: Deutsche med. Wochenschr., 1906, **32**, 58.

Kraus and Stenitzer: Zeitschr. f. Immunität., orig., 1909, **3**, 646.

Aronson: Berl. Klin. Woch., 1907, **44**, 572.

Yamanouchi: Compt. Rend. de la Soc. de Biol., 1909, **66**, 1051.

MacFadyen and Rowland: Central. f. Bakt., orig., 1901, **30**, 753.
MacFadyen: Central. f. Bakt., orig., 1906, **41**, 266.
Meyer and Bergell: Berl. Klin. Woch., 1907, **44**, 568.
Kraus and Stenitzer: Wien. Klin. Woch., 1907, **20**, 344.
Brau and Denier: Compt. Rend. de la Soc. de Biol., 1904, **56**, 433.
Brau and Denier: Ann. de l'Inst. Pasteur., 1906, **20**, 578.
Kraus; **Kolle and Wassermann** Handbuch, vol. 1, p. 180.
MacFadyen: Central. f. Bakt., orig., 1906, **42**, 365.
Metchnikoff, Roux and Salimbeni: Ann. de l'Inst. Pasteur, 1896, **10**, 257.
Doerr, Kraus and Levaditi Handbuch, vol. 1, p. 145.
Kraus and Doerr; **Kraus and Levaditi** Handbuch, vol. 2.
Kraus and Doerr; Zeit. f. Hyg., 1906, **55**, 1.
Todd: British Med. Journ., 1903, **2**, 1456.
Olitsky and Kligler: Journ. of Exper. Med., 1920, **31**, 19.
Pfeiffer and Ungermann: Central. f. Bakt., Orig., 1909, vol. **50**,
Bessau: Central. f. Bakt., 1911, **57**, 27.
Marmorek: Ann. de l'Inst. Pasteur, 1902, **16**, 169.
Braun: Central f. Bakt., 1912, **62**, 383.
Aronson: Berlin. Klin. Woch., 1902, **39**, 979, 1006.
Simons: Central. f. Bakt., Orig., 1904, **35**, 308, 440.
Clark and Felton: Journ. of the Amer. Med. Assoc., 1918, **71**, 1048.

STUDIES IN ANAPHYLAXIS

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I. ON THE QUANTITATIVE REACTION OF PARTIALLY NEUTRALIZED PRECIPITIN IN VITRO AND IN VIVO

In one of the numerous studies (1) with which Richard Weil enriched the literature of anaphylaxis he came to the conclusion that the reaction between antigen and antibody that occurs within or upon the susceptible cells in the anaphylactic guinea-pig takes place in a manner different from that of the reaction of these two substances *in vitro*. This conclusion was drawn from a quantitative study of the anaphylactic reactivity of passively sensitized guinea-pigs that had been partially desensitized.

Weil states that when he passively sensitized guinea-pigs with different amounts of immune rabbit's serum and partially desensitized these different animals with the same quantity of the antigen he found that the animals were all equally sensitive to a further injection of the antigen; the "minimal anaphylactic dose" of the antigen was the same for all of them. The result was the same when guinea-pigs passively sensitized with the same amount of the immune serum were partially desensitized with varying amounts of the antigen; with all of these animals, also, the "minimal anaphylactic dose" of the antigen was the same.

These experiments, which are summarized in Weil's tables 12 and 17, revealed a second peculiarity which forced Weil to the conclusion that "partially desensitized (or neutralized) antibody reacts to antigen in a manner which is quite different from that of pure antibody." This peculiarity lies in the fact that the minimal anaphylactic dose of the antigen was much greater for the partially desensitized animals—0.5 cc.—than it was for the animals that had been sensitized with any smaller dose of immune serum, but without being partially desensitized—0.005 to 0.05 cc. According to Weil this latter phenomenon made it seem evident "that desensitization cannot be explained on the basis of the neutralization or saturation of a fraction of the cellular antibody."

Weil wrote that these two phenomena have no counterpart in the precipitin reaction *in vitro* nor, indeed, "in immunological science." However, since he did not attempt to reproduce the phenomena in the test tube, we have taken up this question experimentally in the present study.

The usual plan of our investigation was, on the one hand, to sensitize guinea-pigs passively with a precipitating immune rabbit's serum and, after partial desensitization with varying amounts of the antigen, to determine the minimal fatal dose of the latter in the partially desensitized animals; on the other hand we mixed in test tubes quantities of the immune serum and antigen that corresponded with those used in these animal experiments and after removal of the resulting precipitate by centrifugation we determined the minimal precipitating amount of the antigen with the supernatant fluid. The supernatant fluid was also injected into a series of guinea-pigs and the minimal lethal dose of the antigen was determined for the animals so treated.

By a comparison of the results of these parallel tests, it can be seen whether the interaction of precipitin and precipitinogen is different *in vivo* and *in vitro* as Weil thought.

Such experiments were carried out with the pseudoglobulin[1] of horse-serum and with crystalline albumin prepared from the white of hen's egg.

The immune serum used in the first experiment was a mixture of sera derived from two rabbits (170 and 397) that had received numerous intraperitoneal injections of crystallline egg albumin, as follows: 2 cc., 10 cc. and 5 cc. on the first, fifth and eleventh days, and 0.5 cc. daily from the thirteenth to the twenty-sixth days inclusive. The animals were bled four days after the last injection.

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[1] For a generous supply of this material we are indebted to Charles R. Tyler, Research and Antitoxin Laboratory, Department of Health of the City of New York, Otisville, N. Y.

The minimal sensitizing dose[2] of this serum mixture was found to be 0.2 cc.

The minimal anaphylactic dose of the egg albumin was determined for guinea-pigs that had been sensitized twenty-four hours previously with 0.4 cc. of the serum mixture. The protocol of this determination is presented in table 1.

TABLE 1

Determination of the minimal lethal dose of egg-albumin after a sensitizing dose of 0.4 cc. of serum 170+397

GUINEA-PIG	EGG-ALBUMIN, INTRAVENOUSLY	RESULT
	cc.	
1	0.0025	Slight symptoms*
2	0.005	Severe symptoms
3	0.0075	Moderate symptoms
4	0.01	✠
5	0.01	✠

*✠ = typical anaphylactic death within 2½ to 5 minutes.
Severe symptoms = immediate violent convulsions with eventual recovery.
Moderate symptoms = slight to moderate convulsions usually beginning after 4 to 5 minutes.
Slight symptoms = marked dyspnoea; animal lies down on side; no convulsions.

The minimal lethal dose of the albumin was found to be 0.01 cc. 5 cc. of the immune serum (170 and 397) were then mixed with 0.01 cc. of the crystalline egg albumin solution and after two hours at 56°C. the clear supernatant fluid, which was separated from the precipitate by centrifugation, was placed in the ice-box. On the following day, no further precipitation having taken place, the fluid was compared with the untreated serum as to its precipitin titer and as to its sensitizing function.

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[2] In all of these experiments guinea-pigs weighing between 250 and 340 grams were used.

TABLE 4

Determination of the minimal anaphylactic dose of egg-albumin after partial desensitization of passively sensitized guinea-pigs. Sensitization: with 0.4 cc. of serum 170+397. Partial desensitization on the third day: 0.008 cc. of egg-albumin intraperitoneally. Test on the fourth day

GUINEA-PIG	EGG-ALBUMIN, INTRAVENOUSLY cc.	RESULT
1	0.04	No symptoms
2	0.1	Severe symptoms
3	0.1	Very severe symptoms
4	0.2	Very severe symptoms
5	0.2	✠

It is seen that the lethal dose of the antigen by the partial desensitization method of Weil is relatively the same as it is after the corresponding partial neutralization *in vitro.*

In a further series of animals the procedure was the same as that of the preceding partial desensitization experiment except that 0.8 cc. instead of 0.4 cc. of the immune serum were used for the passive sensitization.

The results of the test in this series are shown in table 5.

TABLE 5

Determination of the minimal anaphylactic dose of egg-albumin after partial desensitization of passively sensitized guinea-pigs. Sensitization: with 0.8 cc. of serum 170+397. Desensitization on the third day: 0.008 cc. of egg-albumin intraperitoneally. Test on the fourth day

GUINEA-PIG	EGG-ALBUMIN, INTRAVENOUSLY cc.	RESULT
1	0.1	Moderate symptoms
2	0.1	Moderate symptoms
3	0.1	✠
4	0.1	✠
5	0.2	✠

It is seen that the animals used in the two tests presented in tables 4 and 5 responded too irregularly to allow a satisfactory comparison of the results in the two series although, on the whole, the animals of table 5 appear to have been more sensitive than those of table 4.

For the reader's convenience the results of the foregoing anaphylaxis experiments are summarized in table 6.

TABLE 6

Summary of the foregoing experiments

SENSITIZING DOSE SERUM 170 + 397	NEUTRALIZING DOSE EGG-ALBUMIN		MINIMAL ANAPHYLACTIC DOSE OF EGG-ALBUMIN
	In vitro	In vivo	
cc.	*cc.*	*cc.*	*cc.*
0.4			0.01
0.4	0.0008		0.02–0.03
0.4		0.008	0.2 –0.3
0.8		0.008	0.1 –0.2

In table 2 are summarized the parallel experiments in partial neutralization of the precipitin *in vitro*. The results of these latter tests show that the minimal precipitating quantity of the antigen increases exactly in proportion to the amount of the antigen which is used in the partial neutralization.

The animal and test-tube experiments taken together indicate that, whether the partial neutralization is carried out in the test tube or in the guinea-pigs, the quantitative relationship between antigen and partly neutralized precipitin is the same *in vivo* and *in vitro*.

A second series of experiments was carried out with the pooled sera of two rabbits (425 and 426), both of which had received three intraperitoneal injections of whole egg white as follows: On the first day 5 cc.; on the fifth and tenth days 10 cc. each time. The rabbits were bled on the seventeenth day. In a preliminary test 0.1 cc. of each of these sera had been found capable of fully sensitizing a guinea-pig of about 325 grams weight. Smaller amounts of the sera were not tested.

As in the previous experiments we first determined the minimal anaphylactic dose of the crystalline egg albumin solution for guinea-pigs highly sensitized with the immune rabbits' serum; that is, with 0.3 cc. of the pooled serum 425 and 426. The results of this determination are presented in table 7.

The minimal anaphylactic dose is seen to have been a little more than 0.002 cc. of our crystalline albumin solution.

Three further series of animals were sensitized each with 0.3 cc. of the serum 425 and 426 and, after a partial desensitization with the egg albumin solution in amounts differing in the three series, the minimal anaphylactic dose was determined. The results of this experiment are shown in table 8-a, b and c.

Here again the minimal anaphylactic dose of the antigen increases in exact proportion to the increase in the amount of the antigen used for the partial desensitization (0.00025: 0.0025:: 0.035 : 0.35).

TABLE 7

Determination of the minimal anaphylactic dose of antigen after passive sensitization with the untreated immune rabbits' serum. Sensitization: with 0.3 cc. of the pooled serum (425+426)

GUINEA-PIG	EGG-ALBUMIN, INTRAVENOUSLY	RESULT
	cc.	
1	0.00075	No symptoms
2	0.0015	Mild symptoms
3	0.002	Severe symptoms
4	0.002	✠
5	0.005	✠

It is seen that the injection of 0.01 cc. of our solution of crystalline egg albumin completely desensitized the animals. This result is in quantitative disagreement with those of Weil.[4] For although Weil's animals were sensitized with approximately the same amount of precipitating serum as ours (0.2 to 0.4 cc.) and although they were equally sensitive to the antigen (0.001 cc. of Weil's 5 per cent solution as compared with 0.002 cc. of our 3 per cent solution) they were only partially desensitized with 0.01 cc. and 0.04 cc. of the 5 per cent solution of the antigen. Our five animals were all completely desensitized with 0.01 cc. of a 3 per cent solution of the antigen.

With the serum mixture 425–426 partial neutralization *in vitro* was carried out exactly as in the experiments with serum mixture 170–397.

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[4] See Weil's table 18.

The results of this experiment are presented in table 9.

It appears from these tests that after the immune serum 425–426 had been partially neutralized with 0.0025 cc. of the antigen the minimal precipitating dose of the antigen was about ten times as great as it was after a partial neutralization with 0.00025 cc. of the antigen. Corresponding with the animal experiment, 0.01 cc. of the antigen completely neutralized the precipitin in 0.3 cc. of the immune serum.

TABLE 8

Determination of the minimal anaphylactic dose of antigen after partial desensitization (in vivo)

GUINEA-PIG	EGG-ALBUMIN, INTRAVENOUSLY	RESULT
a. Sensitization: with 0.3 cc. of serum (425+426). Partial desensitization on the third day with crystalline egg-albumin, 0.00025 cc. intraperitoneally.		
	cc.	
1	0.0133	Slight symptoms
2	0.02	Severe symptoms
3	0.025	Slight symptoms
4	0.03	Severe symptoms
5	0.035	✠
b. Sensitization: with 0.3 cc. of serum (425+426). Partial desensitization on the third day with crystalline egg-albumin, 0.0025 cc. intraperitoneally.		
1	0.2	Slight symptoms
2	0.3	Mild symptoms
3	0.3	✠
4	0.35	✠
5	0.35	✠
c. Sensitization: with 0.3 cc. of serum (425+426). Desensitization on the third day with crystalline egg-albumin; 0.01 cc. intraperitoneally.		
1	1.0	No symptoms
2	1.0	No symptoms
3	2.0	No symptoms
4	2.0	No symptoms
5	2.0	No symptoms

The results of the parallel tests with the method of complement fixation were in satisfactory agreement with those of the precipitin tests.

TABLE 9

Comparison of the precipitation titer and complement-fixation titer of the original and the partially neutralized serum (425+426). Partial neutralization same as before

PROPORTION OF THE IMMUNE SERUM AND ANTIGEN MIXED		TITER OF THE PRECIPITATION AND COMPLEMENT-FIXATION															
Serum	Antigen (egg-albumin) 3 per cent	Antigen..........	0.05	0.02	0.01	0.005	0.002	0.001	0.0005	0.0002	0.0001	0.00005	0.00002	0.00001	0.000005	0.000002	0.000001
cc.	cc.																
Un-treated		Precipitation	–							+++	++	++	+(+)	+	±	±	–
		Complement-fixation								0	0	0	0	mod.	C	C	C
0.3	0.00025	Precipitation								++	+(+)	+	±	±	–	–	–
		Complement-fixation								0	0	mod.	C	C	C	C	C
0.3	0.0025	Precipitation	–	+	+(+)	++	++(+)	++	+(+)	±	–	–	–	–	–	–	–
		Complement-fixation				0	0	0	weak	C	C	C	C	C	C	C	C
0.3	0.1	Precipitation	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
		Complement-fixation	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C

One quantitative discrepancy is apparent between the results of the animal experiment and those of the test tube experiment. The ratio between the normal minimal anaphylactic dose of the antigen (0.002 cc.) and that after partial desensitization with 0.00025 cc. of the egg albumin solution (0.035 cc.) is not the same as the ratio between the normal minimal precipitating dose of the antigen (0.000002 cc.) and that after partial neutralization *in vitro* with 0.00025 cc. (0.00001 cc.).

This discrepancy could be largely explained by a retest of the immune serum on the seventh day after it had been obtained from the rabbits. At this retest it was found that the minimal anaphylactic dose of the antigen for guinea-pigs passively sensitized with 0.3 cc. of the immune serum had increased to at least 0.005 cc.[5] A retest of immune serum 170–397 after it had been kept for one month in the ice-box showed a similar increase in the minimal anaphylactic dose of the antigen; that is from 0.01 cc. to 0.02 cc.

As the desensitization experiments had been carried out on the fifth or sixth days after the serum had been drawn, it is likely that the minimal anaphylactic dose of antigen had already increased.

The experiments with horse serum pseudoglobulin were carried out with the pooled sera of two rabbits (322 and 388) which had received two intraperitoneal injections of pseudoglobulin as follows: first day 1 cc.; fifth day 5 cc. The rabbits were bled seven days after the second injection.

The minimal sensitizing dose of the pooled sera was found to be 0.8 cc. The minimal anaphylactic dose of the antigen for guinea-pigs that had been sensitized with four doses of the pooled immune sera 322–388 was found, as is seen in table 10, to be 0.02 cc.

In two series of animals we studied the effect of partial neutralization of the precipitin on the minimal anaphylactic dose of antigen. In the first series of animals the partial neutralization was carried out in the test tube and the supernatant fluid obtained after the partial precipitation was tested with the anaphylaxis reaction and also with the precipitin reaction and the complement fixation method *in vitro*.

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[5] 0.004 cc. produced only slight symptoms; 0.005 cc. killed the only animal tested.

TABLE 10

Determination of the minimal anaphylactic dose of pseudoglobulin after passive sensitization. Sensitization: with 3.2 cc. of serum 322+388.

GUINEA-PIG	PSEUDOGLOBULIN OF HORSE SERUM INTRAVENOUSLY INJECTED	RESULT
	cc.	
1	0.01	Very severe symptoms
2	0.015	Very severe symptoms
3	0.02	✠

The results of these parallel tests are presented in tables 11 and 13.

TABLE 11

Determination of the minimal anaphylactic dose of antigen after partial neutralization of the precipitin (in vitro). 18 cc. serum (322+388)+0.4 cc. pseudoglobulin (1–56). After two hours in the incubator and twenty hours at room temperature, the mixture was centrifuged and the supernatant fluid was used for sensitization. 3.2 cc. of the fluid was injected intraperitoneally into each animal. The tests were made on the following day

GUINEA-PIG	PSEUDOGLOBULIN OF HORSE SERUM, INTRAVENOUSLY	RESULT
	cc.	
1	0.05	Very slight symptoms
2	0.1	Severe symptoms
3	0.1	Death (delayed shock)
4	0.15	✠
5	0.15	✠

TABLE 12

Determination of the minimal anaphylactic dose after partial desensitization. Sensitization: with 3.2 cc. of serum 322+388. Partial desensitization: on the third day with 0.284 cc. of pseudoglobulin (1–112), intraperitoneally.

GUINEA-PIG	PSEUDOGLOBULIN OF HORSE SERUM, INTRAVENOUSLY	RESULT
	cc.	
1	0.2	Moderate symptoms
2	0.3	Moderate symptoms
3	0.3	Moderate symptoms
4	0.4	Very severe symptoms
5	0.4	✠

TABLE 13

Comparison of the precipitation titer of the original and the partially neutralized serum (322+388). Partial neutralization in vitro: serum and antigen (pseudoglobulin of horse serum) are mixed well and incubated 4 hours and then left twenty hours in room temperature. The mixture is centrifuged and the supernatant fluid is tested

PROPORTION OF THE IMMUNE SERUM AND ANTIGEN MIXED		TITER OF THE PRECIPITATION (ANTISERUM 0.1 CC. IN EACH TUBE)																		
Serum	Antigen (pseudoglobulin)	Antigen	0.2	0.1	0.05	0.02	0.01	0.005	0.002	0.001	0.0005	0.0002	0.0001	0.00005	0.00002	0.00001	0.000005	0.000002	0.000001
(1) 18 cc. (3.2)	0.4 cc. (1–56) (0.00127)	Precipitation					+++	+++	+++	+++	++	++	+	±	±	–	–	–	–
(2) Untreated	serum	Precipitation					+++	++++	++++	++++	+++	+++	+++	++	++	+	±	±	–

It is seen that the minimal anaphylactic dose of the antigen was increased by the partial neutralization about 7½ fold, while the minimal precipitating amount of the antigen was increased apparently about 10 fold. This slight discrepancy is, no doubt, due to the inherent inaccuracy in the precipitation method.

In another series of animals which had been passively sensitized with the untreated serum mixture 322–388, the effect of partial desensitization on the minimal anaphylactic dose of the antigen was determined.

The results of this determination are presented in table 12.

The amount of antigen used for the partial desensitization was relatively twice that used for the preceding partial neutralization experiment.

It is seen that in this experiment the minimal anaphylactic dose of the antigen was somewhat more than twice that after the partial neutralization experiment. This apparent discrepancy can be explained in the same way as those previously noted. In this instance the immune serum was four days old at the time of the test-tube experiment and seven days old at the time of the desensitization experiment, at which time, therefore, the minimal anaphylactic dose of the antigen had increased for the untreated immune serum.

All of the experiments that have been described above point to the conclusion that precipitin remains unaltered quantitatively and qualitatively in the guinea-pig for several days and that it reacts in the animal body with its antigen in exactly the same manner as it does in the test tube. Furthermore, the experiments offer additional evidence pointing to the identity of precipitating and sensitizing antibody.

II. ON ANTISENSITIZATION

A condition of resistance to passive sensitization with a heterologous immune serum was observed by Richard Weil to result from the previous injection of the normal heterologous serum and this condition was designated by Weil as "antisensitization." The following facts were adduced by Weil regarding the phenomenon:

1. The previous injection of rabbit's serum obstructs the passive sensitization with rabbit's immune serum but not with a homologous (guinea-pig's) immune serum.

2. The interference is established after an incubation period, which is longer (eight days) after small injections (0.1 to 0.5 cc. repeated) than after large injections (1 to 8 cc.); that is, four days.

3. The duration of the normal period of heterologous passive sensitization in guinea-pigs is six days. If the guinea-pigs have received 0.1 cc. of normal rabbit's serum two to eight days previously, the period of passive sensitization with heterologous serum is shortened to barely five days. This period may be shortened, also, by the injection of 0.6 cc. of normal rabbit's serum made on the day following the passively sensitizing injection.

4. The refractory condition of "antisensitization" persists for at least sixty-eight days.

5. Active sensitization is unaffected by the injection of large amounts of normal rabbit's serum.

Weil believed the condition of antisensitization to be due to the interference of antibodies and he supported this belief with the demonstration of an obstructive action on the part of the serum of guinea-pigs that had been immunized with normal rabbit's serum. This view of Weil was apparently contradicted by his own observation that the resistance of antisensitization is not specific; it could be induced by the previous injection of the serum of the sheep and the dog or that of man.

Weil met this difficulty by demonstrating that guinea-pigs sensitized to rabbit's serum are actually hypersensitive to large injections of the three apparently unrelated sera; that is, to sheep's serum, dog's serum and human serum.

As it has been pointed out elsewhere (2), this observation of Weil is in disagreement with those of Ehrlich and Sachs, who reported that their so-called antiamboceptor was specific.

It may be remarked that if Weil's explanation of the apparent non-specificity of his anti-antibodies is to be accepted it must be upon the assumption that the sensitizing antibodies of the immune rabbit's serum are identical, in their antigenic specificity, with the common partial antigens of the three nonrelated sera, an astonishing coincidence.

If, as Weil believed, the phenomenon of antisensitization is due to the action of anti-antibodies, it should be possible to demonstrate a corresponding interference with passive sensitization when the immune serum, previous to its injection, is mixed with an anti-immune serum. Moreover, it should be possible to show that this interference is actually due to the specific precipitation ("neutralization") of the sensitizing antibodies.

With the purpose of applying this test to Weil's theory of the mechanism of antisensitization, we have carried out the succeeding experiments. On April 2, 7 and 12, three normal guinea-pigs received intraperitoneal injections of 0.5 cc., 1 cc. and 1 cc. respectively of the serum of a rabbit that had been highly immunized with egg albumin. On April 18 these three animals were bled to death and the sera obtained from the defibrinated blood were pooled. The mixed serum was tested, as to its precipitating power, with rabbit's serum. The result of this test is shown in table 14.

TABLE 14

Determination of the precipitation titer of the pooled immune guinea-pig serum

IMMUNE GUINEA-PIG'S SERUM IN EACH TUBE	CUBIC CENTIMETERS OF RABBIT SERUM							
	0.1	0.05	0.03	0.01	0.005	0.0025	0.001	1.0005
cc. 0.1	Cloudy	Cloudy	+++	+++	++	+	±	–

With the mixed immune guinea-pigs' serum the "curative experiment" of Weil (3) was carried out as follows: on April 18 three normal guinea-pigs received, by intraperitoneal injection, 0.3 cc. each of the serum of rabbit 440. (This rabbit had been injected a number of times with egg white; the minimal sensitizing dose of its serum was 0.1 cc.) On April 19 each of the three passively sensitized guinea-pigs was given 2 cc. of the pooled immune guinea-pigs' serum by intravenous injection and two days later the three animals were tested as to their hypersensitiveness to egg-albumin by intravenous injection. Two animals responded with very slight symptoms to injections of 1.0 cc. and 0.4 cc. respectively of the undiluted egg albumin solution;[6] the third animal died with the typical symptoms and anatomical changes of anaphylactic shock, after an injection of 0.6 cc. of the egg-albumin solution. The inflation of the lungs in this animal was not maximal.

This result confirms in a general way that obtained by Weil in the single animal employed in his experiment. It reveals, however, an irregularity in the "curative" action of the immune guinea-pigs' serum, which had escaped Weil and which is difficult to harmonize with Weil's view of the mechanism of antisensitization.

It is difficult, in other words, to explain why, notwithstanding the identical treatment of the three guinea-pigs, the "anti-antibodies" of the immune guinea-pigs' serum should have "neutralized" the antibodies of the sensitizing rabbit's serum in two animals but not in the third.

Having demonstrated the "antisensitizing" action of the mixed anti-rabbit guinea-pigs' serum with the technic of Weil's curative experiment, we were in position to examine the mechanism of that action with the test tube experiments. If, as Weil believed, the phenomenon of antisensitization is due to a specific interference by anti-antibodies, then it must be possible to precipitate the sensitizing antibodies from immune rabbit's serum by mixing the latter with the anti-rabbit guinea-pigs' serum *in vitro;* the antibodies of the rabbit's serum should be found to enter into the resulting specific precipitate as indicated by their absence in the supernatant fluid obtained by centrifugation.

On April 21, 12 cc. of the pooled anti-rabbit guinea-pigs' serum were mixed with 1.8 cc. of the anti-egg albumin serum of rabbit 440. This mixture was kept for one hour at 37°C. and then overnight in the ice-box. The precipitate which had formed was removed by rapid prolonged centrifugation and the clear supernatant fluid was examined: (1) As to its content of rabbit's serum proteins; (2) as to its power passively to sensitize against egg albumin; (3) as to its content of anti-egg albumin precipitins.

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[6] The minimal lethal dose of this solution for guinea-pigs sensitized with three doses of anti-egg albumin immune rabbit's serum had been found to be not more than 0.003 cc.

The examination for the presence of rabbit's serum proteins was made on April 22 by mixing 0.4, 0.2, 0.1, 0.05 and 0.025 cc. of the supernatant fluid with 0.1 cc. of the pooled anti-rabbit guinea-pigs' serum. In none of these mixtures had any precipitation taken place after they had stood overnight. All of the rabbit's serum protein that was precipitable with the anti-rabbit guinea-pigs' serum had been removed.

In examining the supernatant fluid as to its power passively to sensitize against egg albumin, account had to be taken of the dilution of the rabbit's immune serum (approximately 1–8) that had occurred as a result of mixing it with the guinea-pigs' immune serum.

On April 22 a series of normal guinea-pigs were given intraperitoneal injections of 0.8 cc. or 1.6 cc. or 2.3 cc. of the supernatant fluid; that is, amounts of the mixture representing 1, 2 and 3 minimal sensitizing doses of the immune rabbit's serum. On the following day the animals were tested with the intravenous injection of not less than 33 lethal doses of the egg albumin solution. The results of this test are presented in table 15.

TABLE 15

"Antisensitization" in vitro

GUINEA-PIG	AMOUNT OF THE SUPERNATANT FLUID USED FOR SENSITIZATION	AMOUNT OF EGG-ALBUMIN USED FOR TEST-INJECTION	RESULT
	cc.	*cc.*	
1	0.8	0.4	No symptoms
2	1.6	0.4	Slight symptoms
3	2.3	0.4	✠
4	2.3	0.4	✠
5	2.3	0.2	Slight symptoms
6	2.3	0.1	Slight symptoms

These results are susceptible of the interpretation that the effect of the anti-rabbit guinea-pigs' serum had been to increase threefold the sensitizing dose of the immune rabbit's serum and also to increase by more than 66 fold the killing dose of the egg albumin for the passively sensitized guinea-pigs. However, in view of the outcome of our "curative" experiment, it seems at least possible that in the test tube experiment we have encountered the same irregularity of action that we observed in the curative experiment; that is, it is possible that guinea-pigs number 5 and 6 would not have been killed with even 0.4 cc. of the egg albumin solution.

In any case, if Weil's hypothesis were correct it must have been possible to demonstrate, in the supernatant fluid, a change in the precipitating action against egg albumin corresponding with the change in the sensitizing action. In accordance with the results of our experiments in the partial neutralization of precipitin *in vitro* it could be expected that the change in the present instance would consist in an increase of at least 66 fold in the minimal precipitating amount of the egg albumin. However, the examination of the supernatant fluid in this respect showed no such change. Indeed, as the protocol of the comparative titration presented in table 16 shows, precipitation titer of the fluid corresponded exactly with that of the untreated rabbit's immune serum.

TABLE 16

Showing the comparative precipitation titer of the supernatant fluid and the original anti egg white rabbit's serum

	CUBIC CENTIMETERS OF EGG-ALBUMIN			
	0.002	0.0001	0.00002	0.00001
Supernatant fluid in each tube, 0.4 cc	+++	++	+(+)	+
Control (serum 140) in each tube, 0.05 cc	+++	++	+(+)	+

The experiment just concluded demonstrates that the phenomenon of the anti-sensitization is not due to the interference of "anti-antibodies;" the power of the rabbit's precipitin of reacting with egg albumin was not perceptibly altered by contact with the "anti-sensitizing" immune guinea-pigs' serum. Furthermore, the irregularity of the action of the anti-sensitizing guinea-pigs' serum suggests a non-specificity of that action which is in harmony with the observation of Weil that the active form of the anti-sensitization can be induced by the previous injection of heterologous sera.

III. EXPERIMENTS WITH SPECIFIC PRECIPITATES

In one of his studies in anaphylaxis (4) Weil attempted to demonstrate directly the identity of precipitin and sensitizing antibody ("sensitizin"). Weil's procedure was to inject washed specific precipitates into guinea-pigs and to test the injected animals, after a proper interval of time, as to the development of passive sensitization.

In such experiments Weil was usually successful, though in some instances the precipitate failed to sensitize the normal animals.

Our experiences with the partial neutralization of precipitin *in vitro* and *in vivo* made it seem unlikely that the combination of protein and precipitin would be more dissociable in the animal's body after the test-tube reaction than after the reaction that occurs *in vitro*.

We have, therefore, employed Weil's technic in an effort to duplicate his results. According to the procedure in his first experiment a constant amount of the precipitating serum—1.5 cc.—was mixed with different quantities of the antigen, crystalline egg albumin, and after an incubation of one hour at 37°C. and forty-eight hours in the ice-box, the mixtures were centrifugated and the precipitate was twice washed with 2 cc. of sterile saline solution. The whole of the washed precipitate from each mixture was injected intraperitoneally into a normal guinea-pig. Three days later each of the injected guinea-pigs was given a test injection of the egg albumin intravenously. None of the animals exhibited any symptoms of anaphylactic shock. Further details of this experiment are presented in table 17.

TABLE 17

Attempted passive sensitization with washed precipitates

SERUM 322 + 388 (MINIMAL SENSITIZING DOSE 0.1 cc.)	EGG-ALBUMIN	PRECIPITATE	WASHED PRECIPITATE INJECTED INTO GUINEA-PIG ON THIRD DAY	EGG-ALBUMIN INTRAVENOUSLY ON SIXTH DAY	RESULT
cc.	*cc.*			*cc.*	
1.5	0.001	+	1	1.0	No symptoms
1.5	0.005	+++	2	1.0	No symptoms
1.5	0.02	++++	3	0.6	No symptoms

This experiment was repeated with the sera of two rabbits that had been immunized with egg white. The results of these experiments are presented in table 18.

TABLE 18

Attempted passive sensitization with washed specific precipitates

Experiment II. {Serum 440
Serum 419} Immune serum v. egg white

	SERUM 440 MINIMAL (SENSITIZING DOSE) 0.1 cc.	EGG-ALBUMIN	PRECIPITATE	WASHED PRECIPITATE INJECTED INTRAPERITONEALLY INTO GUINEA-PIG ON THIRD DAY	EGG-ALBUMIN INTRAVENOUSLY ON SIXTH DAY	RESULT
	cc.	*cc.*			*cc.*	
Series a	1.5	0.001	+	1	0.5	No symptoms
	1.5	0.005	+++++	2	0.5	No symptoms
	1.5	0.02	++++	3	0.5	Moderate symptoms
	SERUM 440					
Series b	1.5	0.02	++++	4	0.5	No symptoms
	1.5	0.04	++++	5	0.5	No symptoms
	SERUM 419 MINIMAL (SENSITIZING DOSE) 0.1 cc.					
Series c	1.5	0.001	+	6	0.5	No symptoms
	1.5	0.005	+++++	7	0.5	No symptoms
	1.5	0.02	++++	8	0.5	No symptoms

It is seen that a moderate sensitization was attained in only one animal and that when that test was repeated with the precipitate from an identical mixture (series B) not the least sensitization resulted. Thus, it appears that although our one animal may be looked upon as confirming Weil's observation of the passively sensitizing property of specific precipitates, the exhibition of that property is the exception rather than the rule.

The negative results appear the more striking when it is recalled that the precipitates were obtained from ten sensitizing amounts of the immune serum.

In the study of antibodies of various kinds, numerous efforts have been made to separate these from the serum proteins of the immune serum containing those bodies. Such studies have had either a practical end in view or they have sought information as to the chemical nature of the antibodies.

For technical reasons these efforts have been made with antibodies against formed antigens such as bacteria and blood corpuscles and the procedure has consisted in the absorption of the antibodies out of the immune serum with the antigenic elements followed by an extraction of the antigen-antibody combination with various substances at various temperatures. In the extraction fluid nearly all of the experimenters have demonstrated only the antibodies. Weil, alone, reported that he found antigen but no precipitating antibody in his "extracts." Weil mixed horse serum with the anti-horse serum of an immune rabbit and, following Chickering, he extracted the resulting precipitate with 1 per cent sodium carbonate. The extract, when mixed with 0.01 cc. of horse serum, remained clear, but when mixed with 0.5 cc. of the anti-horse serum, it formed a precipitate. This unique result was complicated by the successful *passive* sensitization of normal guinea-pigs, which Weil produced with the same extracts in which he could find no precipitin.

Weil explained this paradoxical outcome with the assumption of a haptophore or sensitizing group and an ergophore or precipitating group in the precipitin molecule.

We have investigated this phenomenon with an anti-egg white rabbit's serum. We mixed the serum with crystalline egg-albumin and dissolved the resulting precipitate, obtained by centrifugation and decantation of the supernatant fluid, in 2 cc. of

1 per cent sodium carbonate. In confirmation of Weil's observation it was found that the addition of 0.5 cc. of rabbit's immune serum did produce a precipitate when mixed with a certain amount of the carbonate solution of the precipitate. However, this effect was shown not to be specific because an equal degree of precipitation could be produced in the carbonate solution of the precipitate with 0.5 cc. of a normal serum or by simply neutralizing the extract. The phenomenon thus represented merely a reprecipitation of a dissolved precipitate and not the specific precipitation of free antigen, as Weil thought.

SUMMARY

1. Experiments are presented which demonstrate that precipitin remains unaltered quantitatively and qualitatively in the guinea-pig for several days and that it reacts in the animal body with its antigen in exactly the same manner as it does in the test tube. The experiments furnish further evidence that precipitin and "sensitizin" are identical.
2. The phenomenon of "anti-sensitization" (Weil) is not due to the action of anti-antibodies. It is a non-specific effect the nature of which is obscure.
3. Passive sensitization with washed specific precipitates has generally failed in our hands.
4. The sodium carbonate extract or solution of specific precipitates do not contain free antigen but represents the whole precipitate in solution.

REFERENCES

(1) **Weil, Richard**: Jour. Immunol., 1917, **2**, 469.
(2) **Tice**: Practice of Medicine, vol. 1, p. 143.
(3) **Weil, Richard**: Zt. f. Immunitatsf., 1914, **23**, 1 (p. 13).
(4) **Weil, Richard**: Jour. Immunol., 1916, **1**, 1.

A STUDY OF THE PRECIPITIN TEST IN CASES OF PNEUMOCOCCUS EMPYEMA

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The large measure of success attendant upon the use of immune sera in the treatment of certain types of pneumococcus pneumonia has given rise to the hope that other infections of similar origin might be benefited.

The invasion of the pleural cavity by the pneumococcus, secondarily to pneumonia, is common—in fact, it is the rule rather than the exception. Whether this infection gives rise to dry pleurisy, or empyema, depends upon the virulence of the micro-organism, the amount of chemiotactic action exerted and the destructive action of elaborated toxins. The very nature of the lymphatic circulation of the pleura with the opportunities for the inflow of immune substances, unquestionably tends to reduce materially the number of secondary cases of empyema. Where, however, the lymph channels are blocked with fibrin and cells as the result of acute pleural infection, there may be a great difference between the amount of circulating antibodies in the body at large and their abundance in the pleural exudation.

Often after the pathological process is well established, neutralization or absorption of all agglutinins, precipitins and bacteriolysins takes place, and the enclosed infection may die out from the effect of the products of cellular degeneration. More often, however, pleural drainage by surgical means is necessary to permit of a rapid interchange of toxin and antitoxin, and bacteria, agglutinin and precipitin.

A study of pleural exudation in empyema is instructive in that it gives a measure of the power of the forces of the body to combat a localized infection.

The crisis of pneumonia is commonly accepted to be the time when the body rides over the infection through its maximum production of immune substances. The crisis of the infection of the pleura in empyema probably takes place in a few instances before surgical intervention, with a resulting sterile empyema—in others with the influx of fresh substances after thoracotomy or of the pointing of empyema necessitans.

Our study of a series of twenty-two cases of empyema has been in the nature of observations on the presence of demonstrable precipitins in the exudation and their fluctuation; and the effect upon them of the introduction of appropriate immune serum. At the same time, the nature of the infection, the entrance of secondary invaders, and the amount of pleural phagocytosis was noted.

These cases were under observation for a period of a few days to several weeks. The action of the serum on the course of the disease was only incidental to other observations, because it was not possible to systematically carry it out to its logical conclusion.

The method of procedure was as follows: Following the operation, pus from the chest was obtained, the type of organism determined, and the presence of precipitin sought for. Subsequently, every other day, when it was possible, the chest cavity was irrigated with sterile saline solution, and 4 ounces of equal parts of appropriate immune serum and saline solution were put into the chest.

This procedure was repeated until the child recovered, or some intercurrent disease interrupted the period of observation. No untoward results were noted from the introduction of the serum.

The type of pneumococcus obtained was chiefly that grouped as I and IV. Only two cases of type II were obtained, and type III organism was not seen. As serum for type IV was not available, only types I and II were treated.

In all of this series of cases the precipitin reaction with the exudate was present following the operation, and, as a rule, continued with increasing positiveness in favorable cases. In certain instances, however, following the injection of considerable quantities of serum, the test occasionally was negative indicating that even following drainage and artificial support all precipitin was absorbed. The amount of phagocytosis and the duration of the presence of pneumococci ran a parallel course with the precipitin reaction. The presence of agglutinins in the exudate proved to be uncertain, and we failed frequently to get satisfactory results. In many instances the pathogenic process was definitely prolonged by the invasion of other organisms than those originally present—such as members of the staphylococcus and diphtheria groups.

Case 1. Boy. Age, thirteen months. Unilateral empyema with history of cold for two weeks before admission.

February 18. Thoracotomy.

February 19. Thick pus received. Smears showed pneumococci and very little phagocytosis. Precipitin test showed the organism to be type II.

February 28. Agglutination test not successful. Chest irrigated and pneumococcus serum, type II, injected into chest cavity. Fairly thick pus obtained. Precipitin test positive. Smears and cultures showed pneumococci, staphylococci and *B. hoffmanii* with not much phagocytosis.

March 8. Chest irrigated and serum injected. Pus obtained. Precipitin test positive. Smears showed few pneumococci, staphylococci and *B. hoffmanii* with increased phagocytosis.

March 13. Child discharged relieved. Very little discharge.

Case 2. Girl. Age, six years. Unilateral empyema with history of pneumonia three and one-half weeks previous.

February 19. Thoracotomy.

February 20. Thick pus obtained. Precipitin test showed organism to be pneumococci type I. Smears showed pneumococci with very little phagocytosis. Agglutination test not successful.

February 25. Chest irrigated and pneumococcus serum type I injected. Pus obtained. Precipitin test positive.

February 26. Chest irrigated and serum injected. Pus obtained. Smears show increased phagocytosis and diminution in the number of pneumococci.

February 27. Button removed from chest.

March 1. Chest irrigated and serum injected.

March 4. Child discharged to Boston City Hospital with measles.

March 16. Child readmitted from Boston City Hospital.

March 18. Child's condition good; wound still draining.

April 6. Child's chest healed. Drain reinserted as temperature had been septic in type for past few days. Thick pus evacuated from chest. Smears show no pneumococci.

April 12. Drain removed.

May 6. Discharged well.

Case 3. Boy. Age, three years. Admitted March 5, 1918. Unilateral empyema with history of pneumonia two weeks previous.

March 5. Thoracotomy.

March 6. Pus obtained. Precipitin test showed organism to be pneumococci type I. Smears showed pneumococci in abundance.

March 8. Chest irrigated and serum type I injected. Pus obtained. Precipitin test positive. Smears and cultures show pneumococci, staphylococcus aureus and albus.

March 11. Chest irrigated and serum injected. Pus obtained. Precipitin test not so clearly positive as at first. Smears showed some phagocytosis and only a few pneumococci.

March 15. Button removed.

March 17. Discharged relieved. Slight drainage from sinus.

Case 4. Boy. Age, six years. Admitted March 20, 1919. Unilateral empyema with history of pneumonia twenty-four days previous.

March 20. Thoracotomy.

March 21. Pus received. Precipitin test showed organism to be pneumococcus, type I. Smears showed pneumococci. Phagocytosis slight.

March 22. Chest irrigated and serum injected.

March 28. Chest irrigated and serum injected. Pus obtained. Smears showed no pneumococci and *B. hoffmanii.*

April 2. Practically no discharge from the chest.

April 7. Cultures taken showed very few pneumococci.

April 9. Child discharged to Boston City Hospital with measles.

April 15. Child readmitted.

April 16. Chest healed.

May 6. Discharged.

Case 5. Girl. Age, five years. Admitted March 22, 1919. Unilateral empyema with history of pneumonia three weeks previous.

March 22. Thoracotomy.

March 23. Heavy pus obtained. Precipitin test showed organism to be pneumococcus, type I. Smears showed pneumococci in chains with little or no phagocytosis.

March 25. Chest irrigated and type I serum injected. Mucopurulent pus obtained. Smears showed a number of pneumococci and slight increase of phagocytosis.

March 28. Chest irrigated and serum injected. Pus obtained. Precipitin test unsatisfactory. Smears and cultures showed a few pneumococci and staphylococcus albus. Phagocytosis more marked.

April 1. Chest irrigated and serum injected. Thick pus obtained. Precipitin test faintly positive. Smears show few pneumococci, numerous bacilli and staphylococci.

April 3. Chest irrigated and serum injected. Button removed. Bloody fluid, no pus, obtained. Precipitin test negative. Cultures showed heavy growth of a diphtheroid, and a few pneumococci.

April 7. Chest irrigated and serum injected. Specimen obtained from chest.

April 8. Cultures showed *B. diphtheroid* and a few colonies of staphylococcus albus.

April 11. Child discharged well.

Case 6. Boy. Age, four years. Admitted March 23, 1919. Unilateral empyema with history of pneumonia three weeks previous.

March 23. Thoracotomy.

March 25. Heavy pus received. Precipitin test showed organism to be pneumococcus, type II. Smears showed a moderate number of pneumococci—some in chains—and no phagocytosis.

March 28. Chest irrigated and serum type II injected. Pus obtained. Smears showed very few pneumococci, well phagocyted, and cultures showed only colonies of *B. xerosis.*

April 1. Chest irrigated and serum injected. Thin pus obtained. Precipitin test negative. Smears showed very few pneumococci, numerous bacilli, probably *B. xerosis.*

April 3. Chest irrigated and serum injected. Button removed. Bloody fluid obtained. Precipitin test negative. Cultures showed very few pneumococci.

April 7. Chest irrigated and serum injected. Mucopurulent fluid obtained. Culture showed an occasional colony of pneumococcus and *B. xerosis* in large numbers.

April 13. Child discharged to Boston City Hospital with measles.

April 20. Child readmitted.

April 26. Marked recrudesence of pus since April 13 Swab showed no pneumococci, and a fair number of typical diphtheria bacilli.

April 28. 1500 units of antitoxin given.

May 14. K. L. bacilli still present.

May 20. Patient improving, but discharge still considerable.

June 3. Discharge lessened.

June 11. Child practically healed. Child discharged.

Case 7. Boy. Age, two years. Admitted April 2, 1919. Unilateral empyema with history of pneumonia one week previous.

April 2. Thoracotomy.

April 3. Thick, heavy, greenish pus received. Precipitin test showed pneumococci to be type I. Smears and cultures showed numerous pneumococci with little or no phagocytosis.

April 7. Chest irrigated and serum type I injected. Pus obtained. Precipitin test markedly positive. Smears and cultures showed pneumococci with great increase of phagocytosis.

April 12. Chest irrigated and serum injected. Very little secretion from chest obtained. Precipitin test negative. Smears show only an occasional pneumococcus. Cultures showed rare colony of pneumococci, and several colonies of staphylococcus albus.

April 16. Chest irrigated and serum injected. Slightly opaque, watery solution obtained. Precipitin test positive. Smears showed almost no organisms and only an occasional leucocyte.

April 18. Temperature septic in type.

May 1. Temperature still septic. Wound draining slightly.

May 7. Cultures from discharge show staphylococcus aureus.

May 15. Temperature still septic. Wound closed.

May 19. Wound reopened and much pus evacuated.

May 29. Septic temperature still present.

May 31. X-ray showed no pus in the chest.

June 19. Child improved. Chest entirely closed.

June 23. Discharged relieved.

Case 8. Girl. Age, five years. Admitted April 11. Unilateral empyema with history of pneumonia twelve days previous.

April 11. Thoracotomy.

April 12. Heavy pus received. Precipitin test slightly positive for type I. Smears and cultures showed numerous pneumococci with a fair amount of phagocytosis. Agglutination test with cultures showed marked positive reaction to serum 1.

April 16. Chest irrigated and serum type I injected. Watery pus obtained. Precipitin test positive. Smears showed a moderate number of pneumococci with a fair amount of phagocytosis. Cultures showed staphylococcus albus.

April 18. Chest irrigated and serum injected. Pus obtained. Precipitin test negative.

April 22. Chest irrigated and serum injected. Fairly heavy pus obtained. Smears showed pneumococci with phagocytosis increased and an increase of staphylococci.

April 26. Chest irrigated and serum injected. Thick greenish pus obtained. Smears showed relatively few pneumococci—many morphologically typical diphtheria bacilli. Cultures showed staphylococcus albus and streptococci.

May 1. Chest irrigated and serum injected. Heavy pus obtained. Precipitin test positive. Smears showed few pneumococci, a considerable number of phagocyted diphtheria bacilli, and staphylococci.

May 6. Chest irrigated and serum injected. Thinner pus obtained. Precipitin test faintly positive. Smears showed moderate number of pneumococci and fairly well phagocyted diphtheria bacilli.

May 8. Chest irrigated and serum injected. Watery pus obtained. Precipitin test negative. Smears showed few pneumococci.

May 16. Diphtheria bacilli still present. Still considerable discharge from chest.

June 8. Wound practically healed. Child discharged.

Case 9. Boy. Age, nine years. Admitted April 20. Unilateral empyema with history of pneumonia three weeks previous.

April 20. Thoracotomy.

April 22. Thick pus received. Precipitin test showed positive reaction to type I serum. Smears showed moderate number of pneumococci, slightly phagocyted.

April 26. Chest irrigated and serum type I injected.

April 27. Button removed.

April 29. Chest irrigated and serum injected. Watery pus received. Precipitin test positive. Smears showed few pneumococci.

May 1. Chest irrigated and serum injected. Thick, yellow pus obtained. Precipitin test positive. Smears showed some pneumococci, well phagocyted. Cultures showed pneumococci and staphylococcus albus.

May 3. Drainage still considerable. Chest irrigated and serum injected. Smears obtained showed few leucocytes, no pneumococci, and few other organisms.

May 6. Chest irrigated and serum injected. Thinner pus obtained. Precipitin test faintly positive. Smears showed few pneumococci with moderate phagocytosis.

May 8. Chest irrigated and serum injected.

May 10. Chest irrigated and serum injected. Pus obtained. Precipitin test faintly positive. Smears show a moderate number of pneumococci well phagocyted.

May 13. Chest irrigated and serum injected. Pus obtained. Precipitin test negative. Smears showed pneumococci in chains and no phagocytosis.

May 15. Chest irrigated and serum injected. Pus obtained. Precipitin test negative. Smears showed broken down leucocytes; few pneumococci; little phagocytosis. Cultures show moderately numerous pneumococci and some streptococci.

May 20. Patient's temperature was septic in type—drainage still profuse. Serum discontinued.

June 11. Temperature normal.

June 16. Child discharged.

Case 10. Boy. Age, nine years. Admitted April 23. Unilateral empyema with history of pneumonia three weeks previous.

April 23. Thoracotomy.

April 24. Pus with heavy film received. Precipitin test showed slight positive reaction with pneumococcus type I. Smears showed many pneumococci; no phagocytosis.

April 26. Chest irrigated and type I serum injected.

April 29. Chest irrigated and serum injected. Thick pus obtained. Precipitin test negative. Smears showed moderate number of pneumococci; slight phagocytosis, many bacilli and staphylococci.

May 3. Chest irrigated and serum injected. Fairly heavy pus obtained. Smears showed few pneumococci, fairly well phagocyted, numerous diphtheroid bacilli and staphylococci.

May 6. Chest irrigated and serum injected. Very little pus obtained. Precipitin test negative. Smears showed few organisms not phagocyted.

May 8. Chest irrigated and serum injected.

May 10. Chest irrigated and serum injected. Watery pus obtained. Precipitin test faintly positive. Smears showed a moderate number of phagocyted pneumococci.

May 13. Chest irrigated and serum injected. Button removed. Very thin pus obtained. Precipitin test positive. Smears showed no organisms.

May 15. Chest irrigated and serum injected. Almost no discharge present. Smears showed few broken down leucocytes and no organisms.

May 16. Child discharged.

Case 11. Boy. Age, four years. Admitted April 26. Bilateral empyema with history of bronchitis four weeks previous.

April 25. Thoracotomy.

April 27. Thick pus obtained. Precipitin test showed positive reaction to type I pneumococcus. Smears and cultures showed pneumococci.

May 3. Chest irrigated and serum type I injected. Heavy pus obtained. Precipitin test positive. Smears showed few pneumococci moderately phagocyted and many diphtheroid bacilli.

May 5. Serum treatment discontinued.

May 6. Second operation.

May 10. Child died.

Case 12. Boy. Age, five years. Admitted April 26. Unilateral empyema with history of pneumonia four weeks previous.

April 26. Thoracotomy.

April 27. Thick pus received. Precipitin test positive for serum type I. Smears and cultures showed pneumococci, but no phagocytosis.

May 1. Chest irrigated and serum type I injected. Watery pus obtained. Precipitin test not satisfactory. Smears and cultures showed a fair number of pneumococci, staphylococcus aureus and albus, and moderate phagocytosis.

May 3. Serum treatment discontinued.

May 9. Child died.

Case 13. Girl. Age, five years. Admitted May 6. Unilateral empyema with history of pneumonia five days previous.

May 6. Thoracotomy.

May 6. Fairly heavy pus received. Precipitin test positive for pneumococcus type I. Smears showed pneumococci with little phagocytosis.

May 13. Chest irrigated and serum I injected. Thin pus obtained. Precipitin test negative. Smears showed scattered pneumococci not phagocyted.

May 15. Chest irrigated and serum injected. Thick pus obtained. Precipitin test negative. Smears showed few pneumococci, mostly phagocyted. Cultures showed numerous colonies of staphylococcus albus, diphtheria bacilli and a few of the pneumococcus.

May 17. Chest irrigated and serum injected. Watery pus obtained. Precipitin test positive.

May 20. Chest irrigated and serum injected. Fairly thick pus obtained. Precipitin test positive. Smears showed fairly numerous pneumococci slightly phagocyted.

May 22. Chest irrigated and serum injected. Thin pus obtained. Precipitin test positive.

May 24. Chest irrigated and serum injected. Thin pus obtained. Precipitin test negative.

May 27. Chest irrigated and serum injected. Thin pus obtained. Precipitin test positive. Smears showed a few chains of pneumococci.

May 29. Chest irrigated and serum injected. Very thin pus obtained. Precipitin test positive. Smears showed no pneumococci.

May 31. Chest irrigated and serum injected—discharge decreased.

June 2. Discharged.

Case 14. Boy. Age, two years. Admitted May 8. Unilateral empyema with history of pneumonia ten days previous.

May 8. Thoracotomy.

May 8. Thick pus received. Precipitin test positive for pneumococcus type I. Smears showed moderate number of pneumococci well phagocyted.

May 13. Chest irrigated and serum injected. Thick pus obtained. Precipitin test positive. Smears showed few pneumococci—no phagocytosis.

May 14. Button removed.

May 15. Chest irrigated and serum injected. Thick pus obtained. Precipitin test negative. Smears showed numerous pneumococci and a fair amount of phagocytosis. Cultures show pneumococci.

May 17. Chest irrigated and serum injected. Thick pus obtained. Precipitin test positive.

May 20. Chest irrigated and serum injected.

May 22. Chest irrigated and serum injected.

May 24. Chest irrigated and serum injected. Very little pus obtained. Precipitin test positive.

May 28. Child discharged.

Case 15. Girl. Age, eight years. Unilateral empyema with history of sickness since measles one year previous.

May 14. Operation (patient has had empyema before). Thoracotomy.

May 15. Thick, bloody pus received. Precipitin test positive for type I pneumococci. Smears showed pneumococci in chains moderately phagocyted. Cultures showed some colonies of staphylococcus albus, and only a few pneumococci.

May 17. Chest irrigated and serum injected. Thick pus obtained. Precipitin test faintly positive.

May 20. Chest irrigated and serum injected. Fair amount of pus obtained. Precipitin test negative. Smears showed many pneumococci well phagocyted.

May 22. Chest irrigated and serum injected. Pus obtained. Precipitin test negative.

May 24. Chest irrigated and serum injected. Pus obtained. Precipitin test negative.

May 27. Chest irrigated and serum injected. Pus obtained. Precipitin test negative. Smears showed few pneumococci and a few diphtheroid bacilli.

May 29. Chest irrigated and serum injected. Fairly thick pus obtained. Pecipitin test faintly positive. Smears showed moderate number of pneumococci, staphylococci and many diphtheroid bacilli.

May 31. Chest irrigated and serum injected. Fairly heavy pus obtained. Precipitin test faintly positive. Smears showed numerous pneumococci with slight phagocytosis.

June 1. Button removed.

June 5. Chest irrigated and serum injected.

June 7. Chest irrigated and serum injected. Fairly thick pus obtained. Precipitin test faintly positive. Smears showed few organisms, mostly pneumococci moderately phagocyted.

June 10. Chest irrigated and serum injected.

June 11. Child running high temperature and her condition poor. Serum discontinued.

June 23. Child much worse, having had no improvement since June 11.

June 28. Child died.

Case 16. Boy. Age, four years. Admitted May 14. Unilateral empyema with history of sickness and cough for two weeks previous.

May 14. Operation. Thoracotomy.

May 15. Thick greenish pus obtained. Precipitin test faintly positive, pneumococcus type I. Smears showed a very few pneumococci. Cultures showed pneumococci in pure culture.

May 17. Chest irrigated and serum injected. Thick pus obtained. Precipitin test slightly positive.

May 20. Chest irrigated and serum injected. Fairly thick pus obtained. Precipitin test positive. Smears showed very few pneumococci well phagocyted and many diphtheroid bacilli also phagocyted.

May 22. Chest irrigated and serum injected. Fair amount of pus obtained. Precipitin test negative. Agglutination test positive.

May 24. Chest irrigated and serum injected. Pus obtained. Precipitin test negative.

May 27. Chest irrigated and serum injected. Pus obtained. Precipitin test positive. Smears showed almost no organisms.

May 29. Chest irrigated and serum injected. Pus obtained. Precipitin test positive. Smears showed a few diphtheroid bacilli and practically no pneumococci.

May 31. Chest irrigated and serum injected.

June 3. Button removed.

June 5. Chest irrigated and serum injected. Very thin pus obtained. Precipitin test faintly positive. Smears showed some staphylococci and diphtheroid bacilli.

June 7. Chest irrigated and serum injected. Very thin pus obtained. Precipitin test faintly positive. Smears showed many staphylococci and diphtheroid bacilli, and a few pneumococci.

June 11. Child died.

Case 17. Girl. Age, thirteen months. Admitted June 3. Unilateral empyema with history of pneumonia four weeks previous.

June 3. Operation.

June 4. Thick pus obtained. Precipitin test showed positive reaction to pneumococcus type I. Smears and cultures showed pneumococci, staphylococcus aureus and unidentified bacilli.

June 7. Chest irrigated and serum injected. Pus obtained. Precipitin test positive. Smears showed moderate number of pneumococci.

June 10. Chest irrigated and serum injected. Very thin pus obtained. Precipitin test faintly positive.

June 11. Button removed.

June 15. Chest irrigated and serum injected. Practically no pus obtained. Precipitin test negative.

June 16. Chest irrigated and serum injected. Very little pus obtained. Precipitin test positive. Smears showed very few pneumococci.

June 17. Chest irrigated and serum injected.

June 19. Chest irrigated and serum injected. Very little pus obtained. Agglutination test negative. Smears showed few pneumococci, fairly well phagocyted, and no secondary infection.

June 21. Chest irrigated and serum injected. Very little pus obtained. Precipitin test negative. Agglutination test negative. Cultures showed staphylococcus aureus.

June 24. Chest irrigated and serum injected. Very little pus obtained. Chest practically closed. Precipitin test positive. Smears showed a few pneumococci.

June 28. Chest closed.

July 9. Discharged.

Case 18. Boy. Age, twenty months. Admitted June 4. Unilateral empyema with history of pneumonia three weeks previous.

June 4. Thoracotomy.

June 5. Thick pus received. Precipitin test positive with pneumococcus type I. Smears showed a few pneumococci only slightly phagocyted.

June 7. Chest irrigated and serum injected. Pus obtained. Precipitin test positive. Agglutination test positive. Smears showed a few organisms, mostly bacilli with a few pneumococci.

June 10. Chest irrigated and serum injected. Very little pus obtained. Precipitin test faintly positive.

June 15. Chest irrigated and serum injected. Little pus obtained.

June 17. Chest irrigated and serum injected.

June 19. Chest irrigated and serum injected. Very little pus obtained. Agglutination test faintly positive. Smears showed moderately numerous pneumococci with marked phagocytosis and no secondary infection.

June 21. Chest irrigated and serum injected. Very little pus obtained. Smears showed no organisms.

July 9. Discharged well.

Case 19. Boy. Age, one year. Admitted June 13. Unilateral empyema with history of pneumonia eighteen days previous.

June 13. Thoracotomy.

June 14. Thick pus received. Precipitin test positive for type I pneumococcus. Smears and cultures show only pneumococci.

June 17. Chest irrigated and serum injected.

June 19. Chest irrigated and serum injected. Fairly thick pus obtained. Agglutination test positive. Smears showed many pneumococci and marked phagocytosis.

June 21. Chest irrigated and serum injected. Very little pus obtained. Smears showed very few organisms. Button removed.

June 24. Chest irrigated and serum injected. Very little pus obtained. Precipitin test positive. Smears showed few pneumococci with considerable phagocytosis.

June 26. Chest irrigated and serum injected. Very little pus obtained. Precipitin test positive. Smears showed very few pneumococci.

June 28. Chest irrigated and serum injected. Very little pus obtained. Precipitin test positive. Serum treatment discontinued.

July 22. Discharged.

Case 20. Boy. Age, two years. Admitted June 18. Unilateral empyema with history of pneumonia four weeks previous.

June 18. Operation. Thoracotomy.

June 19. Thick pus received. Precipitin test positive for type I pneumococcus. Smears showed pneumococci in fair numbers. Little phagocytosis.

June 21. Chest irrigated and serum injected. Thick pus obtained. Smears showed very few pneumococci, mostly phagocyted. Serum treatment discontinued.

July 15. Child died.

CONCLUSIONS

1. The presence of an easily demonstrable amount of precipitin in the exudation of empyema is generally a favorable prognostic sign.
2. The amount of demonstrable agglutinin in the exudation of empyema is often very small.
3. Phagocytosis runs a parallel course with increasing pleural resistance.
4. The pleura will clear itself of a pneumococcus infection in a much shorter time than the average, if the secondary invaders can be excluded.
5. Pleural irrigation with an appropriate immune serum in pneumococcus empyema is suggested as a means of treatment in order to increase locally those immune substances that tend to limit the duration of the infection.

I wish to take this opportunity to thank Dr. William E. Ladd on whose service at the Children's Hospital these cases were observed.

A NEPHELOMETRIC METHOD OF ESTIMATING THE NUMBER OF ORGANISMS IN A VACCINE

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In the manufacture of a bacterial vaccine, the determination of the number of organisms per cubic centimeter is a very important part of the work. For this determination two methods are in general use, the microscopical count and the measurement of the opacity of the bacterial suspension as compared with the opacity of a bacterial or chemical standard. The microscopical count is usually made either by the Wright method or with a Halber counting chamber. The measurement of the opacity, or the turbidity, of a vaccine is made, in the older methods, by comparing the turbidity of a tube of bacterial suspension, of unknown concentration, with that of a series of tubes containing standard suspensions. The standards consist of bacterial suspensions, or of suspensions of a chemical such as barium sulphate, in which the turbidity of each suspension represents a certain bacterial concentration. More recently a method has been devised for estimating the opacity of a suspension by measuring the depth at which a wire loop disappears from view in the suspension as compared with the depth at which it disappears in a suspension of known concentration (1).

Counting the bacteria in a vaccine with the microscope is laborious, time consuming, and in routine work it is liable to considerable error. Estimation of the concentration of a vaccine by the comparative measurement of the turbidity of the opacity of the suspension, by the methods in use at the present time, is subject to error due to the differences in the acuteness of vision of different individuals, and the variations in the color of the suspensions. With the nephelometer the errors caused by the differences in the acuteness of vision of the different observers, and variations in the color of the suspensions, are, to a large extent, eliminated. This reduction in the percentage of error is due to the accuracy with which the depth of the suspension can be measured and the accuracy with which the two fields of reflected light can be matched.

In the performance of this work the Kober (2) nephelometer was used. This instrument was primarily devised for the estimation of the amount of suspended matter in analytical work, such as fats in blood and milk, and the results obtained are based upon the measurement of depth of the suspension that is necessary to reflect the same amount of light as that reflected by a standard suspension at a fixed depth. With this apparatus artificial light passes into the suspension in the cups on either side and is reflected through the plungers and eyepiece to the eye of the observer. The cups can be raised or lowered independently of each other and the extent of this movement is accurately measured on the corresponding vernier millimeter scale on the back of the instrument. By means of the scale the depth of the suspension required to reflect a certain amount of light can be measured directly to 0.1 mm. and by interpolation to 0.01 mm. The arrangement of the cups, plungers and eyepiece gives two flat, side by side, fields of light which can be accurately matched.

Standards consisting of suspensions of bacteria have been found to give the most satisfactory results. These are made by suspending the killed bacteria in normal salt solution to which a small amount of antiseptic has been added. An accurate microscopical count, with the Halber counting chamber, is made of the bacteria in this suspension, which is then diluted until it contains the desired number of bacteria per cubic centimeter. Several recounts should be made.

Standards which consist of material other than bacteria, such as barium sulphate, may be used. However, barium sulphate will settle out of an aqueous suspension too rapidly for accurate work. When it is suspended in glycerin, or a similar substance of sufficient density to prevent this settling, the air bubbles remain in the fluid for some time and interfere with the readings. On the other hand, bacterial suspensions will remain accurate in the instrument for from twenty to thirty minutes and then only require to be shaken.

This work has been done in connection with the preparation of monovalent typhoid and paratyphoid suspensions in the manufacture of triple typhoid vaccines. Monovalent standards have been used in which the standard for the paratyphoid A vaccine contained two billion bacilli per cubic centimeter, that for the paratyphoid B vaccine contained two billion bacilli per cubic centimeter, and the standard for the typhoid vaccine contained one billion bacilli per cubic centimeter.

In order to determine the number of bacteria in a vaccine of unknown concentration, 10 to 12 cc. of the standard for that vaccine are placed in the cup on the left side of the instrument. This cup is then raised until the vernier scale on the left side is set at 20 mm. indicating that the column of the standard suspension between the bottom of the plunger and the bottom of the cup is 20 mm. in depth. A proper dilution of the vaccine to be counted is then placed in the cup on the right side. This cup is raised until the light that is reflected through the eyepiece from the bacteria in the two suspensions is exactly equal on both sides of the field. The depth of the suspension of the unknown vaccine, that is required to reflect the same amount of light as 20 mm. of the standard suspension, can then be read on the right vernier scale.

The concentration of the unknown suspension is not exactly inversely proportional to readings of the scale. To supply the necessary correction Kober has suggested the following equations (3):

$$X = \frac{S + Sk + \sqrt{(S + Sk)^2 - 4SkY}}{2Y} \text{ or } Y = \frac{S}{X} - \frac{(I - X)Sk}{X^2}$$

In which, Y = the reading of the unknown on the millimeter scale.

S = the reading of the standard on the millimeter scale.

X = the ratio of the concentration of the two suspensions.

$k = \frac{K}{S}$ where K = a constant obtained by the substitution of the standardization values of S, Y, and X.

To use the formula for each calculation would, however, involve a considerable expenditure of time. It is much more practicable to use a nephelometeric curve. To make this curve, the left cup containing the standard is set at 20 mm. Various dilutions, such as 0.9, 0.8, 0.7, and 0.6, of the standard are read against the undiluted standard. The readings thus obtained are plotted on cross section paper and a curve drawn through the established points (fig. 1). Once this curve has been plotted, it may be used for all future work with that standard. Before each series of readings, the standard should be read against itself and the vernier scale on the right adjusted so that the light reflected from one suspension matches that reflected from the other when both scales are set at 20 mm. When this is done, the original curve is applicable.

It is necessary to dilute the vaccine to be counted so that the readings will come within the limits of the curve.

As all parts of the curve represent a known number of bacteria per cubic centimeter, it is only necessary to apply the reading of the millimeter scale to the curve to obtain the number of bacteria per cubic centimeter in the dilution that is being read. This result multiplied by the dilution will equal the number of bacteria per cubic centimeter in the undiluted vaccine. For example, assuming that a paratyphoid A vaccine, diluted to one part in four, gives a reading of 24.7 mm. on the scale, reference to the curve for this vaccine (fig. 1) shows that this reading represents 0.75 of the concentration of the standard (2000 million bacilli per cubic centimeter) or 1500 million bacilli per cubic centimeter. This figure multiplied by four gives 6000 million bacilli per cubic centimeter, which is the concentration of the original vaccine.

The bacterial standards should be renewed each month. A fresh vaccine is diluted until it exactly matches the old standard in the nephelometer. This procedure requires only a short time and avoids any danger of autolysis. Standards three months old have been found to be accurate.

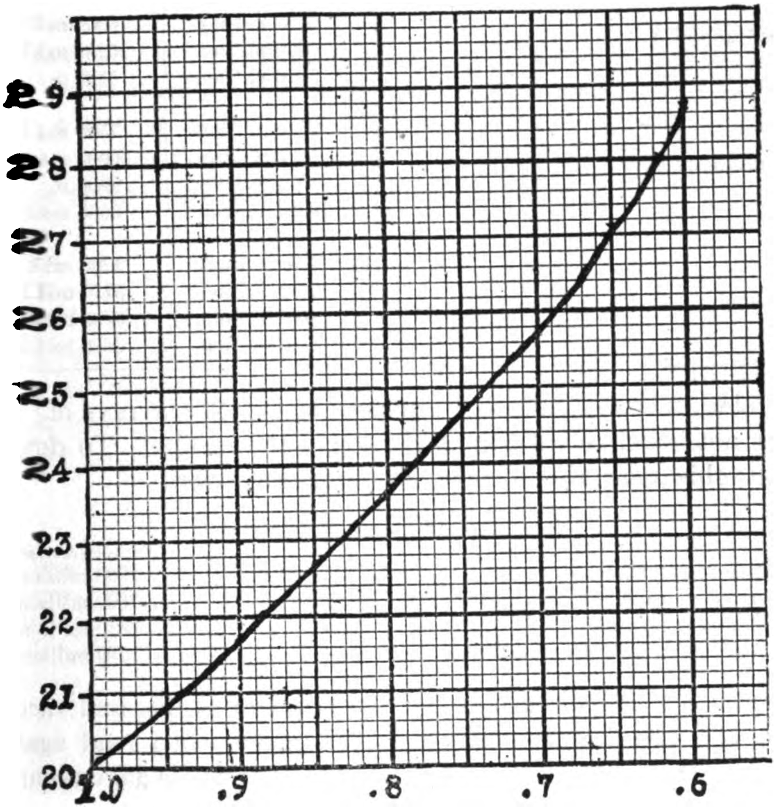

Fig. 1. Nephelometric Curve for the Paratyphoid—A Suspension

Concentration of the standard, 2000 million bacilli per cubic centimeter. The ordinates represent the millimeter readings on the scale. The abscissae represent the ratio of the concentration of the unknown suspension to that of the standard.

1.0 = 2000 million bacilli per cubic centimeter
0.9 = 1800 million bacilli per cubic centimeter
0.8 = 1600 million bacilli per cubic centimeter
0.7 = 1400 million bacilli per cubic centimeter
0.6 = 1200 million bacilli per cubic centimeter

The results that are outlined below are typical of those obtained by this method.

VACCINE	NEPHELOMETRIC ESTIMATION PER CUBIC CENTIMETER	MICROSCOPICAL COUNT PER CUBIC CENTIMETER
Paratyphoid A	5440 million	5600 million
	6080 million	6000 million
	5220 million	5280 million
	5070 million	5120 million
Paratyphoid B	7320 million	7500 million
	9500 million	10000 million
	9200 million	9000 million
	10000 million	9600 million
Typhoid	4236 million	4200 million
	3440 million	3420 million
	3700 million	3760 million
	3780 million	3520 million

Color, unless of sufficient intensity to cut off the rays of light, does not interfere with the accuracy of the results. To demonstrate this point, the following examples are given:

Read against the usual standard

	Per cubic centimeter
Before coloring	7800 million
After coloring with methylene blue	7600 million
Before coloring	7080 million
After coloring with picric acid	7080 million

Vaccine that had been grown on dark agar media, and vaccine that had been grown on blood agar media when read against a standard that had been grown on light agar media gave accurate results. For example:

	NEPHELOMETRIC ESTIMATION PER CUBIC CENTIMETER	MICROSCOPICAL COUNT PER CUBIC CENTIMETER
Typhoid (dark agar)	3480 million	3520 million
Typhoid (blood agar)	2670 million	2640 million

It has been found that certain cultures of paratyphoid B take the stain very poorly and also that some of the cultures exhibit a tendency towards fragmentation. These factors render the bacilli very difficult to count and introduce a considerable error into the microscopical count. With the nephelometer the error due to the poor staining qualities is eliminated. The fragmented bacilli reflect the light in proportion to their mass as well as the whole bacilli.

In general, the advantages of the nephelometric method over the microscopical are the saving of time and labor. The average reading can be made and calculated in less than ten minutes. The error should not be greater than 10 per cent plus or minus. This is as accurate as the microscopical count, if not more so.

SUMMARY

A method is described for estimating the number of organisms per cubic centimeter in a vaccine by means of the nephelometer.

The estimation can be made in less time and with less labor than a microscopical count, and the results are fully as accurate as those obtained with the microscope.

REFERENCES

(1) Gates: Jour. Exp. Med., 1920, **29**, 105.
(2) Kober: Jour. Biol. Chem., 1917, **29**, 155.
(3) Kober: Jour. Am. Chem. Soc., 1915, **37**, 2379. Jour. Biol. Chem., 1913, **13**, 485.

EFFECT OF ULTRAVIOLET RAYS ON ANTIGENIC PROPERTIES

I. STUDIES ON MENINGOCOCCUS

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As early as 1885, Duclaux (1) showed that light exerted a destructive action on spores. His work was followed by the researches of Arloing (2) who studied particularly the *B. anthracis*. Koch (3) in 1890, applied these results to the tubercle bacillus and succeeded in demonstrating the same principles. Confirmatory studies were published by Mignesco (4) in 1896.

The bactericidal power of sunlight is not dependent upon heat but as Ward (5) has reported, is a function of the ultraviolet rays. Indeed, the earliest studies on the effect of sunlight upon bacteria suspended in water (6) tend to disprove the idea that prolonged heating is responsible for the destruction.

The rôle which photochemical action of light plays in the phenomenon is not definitely established. Peroxide of hydrogen may be formed in fluid media under aerobic conditions, yet the action of ultraviolet rays is too marked to be explained in this manner. In addition to this, there is direct evidence that dried bacteria are similarly influenced (7). On the other hand, much has been written on the photodynamic influence of fluorescent substances on microörganisms and the analogy between the action of light and photodynamics presented. The studies of Jodlbauer and Tappeiner (8), Tappeiner (9) and Finssen (10) contain pertinent data. Contradictory evidence is given, however, by Cernovodeanu and Henri (11) who contend that pigmented as well as unpigmented organisms do not differ in their resistance to ultraviolet rays. Later work by Burge (12) compared the rate of death of fluorescent and non-fluorescent (12) bacteria when subjected to ultraviolet light and advanced the theory that coagulation of bacterial protoplasm was responsible for the bactericidal effect.

More recently, the literature on ultraviolet rays has been devoted to the practical application of water sterilization. Buchner (13) in 1893, presented data which paved the way for later studies among which may be mentioned those of Desfosses (14) and Nagier (15).

Apparently no attempt has been made to study the action of the ultraviolet ray from the standpoint of bacterial attenuation or physicochemical modification. In this paper the problem has been approached from these angles and an effort made to rule out the factor of heat in the experiments, so that no extraneous influence other than that of the ultraviolet ray might be involved.

EXPERIMENTAL

The most suitable organisms for a study of this kind are those which belong to a group characterized by a serologic relationship of some of its species. The effect of any external influence such as ultraviolet light ought to be manifested readily owing to the unusual susceptibility to change which any transitional group of organisms possess. These experiments must be considered as secondary in importance to the problem for which they were intended; namely, that of finding a method for producing potent sera and vaccines by the use of living cultures, without the usual untoward manifestations which attend present methods. For such a study any group of organisms may suffice. We have employed the pneumococcus and have found that the principles laid down as a result of the experiments with meningococci are applicable to the pneumococcus and that it is possible to use living organisms for active immunization with great success.

It is essential in studying the action of the ultraviolet ray to suspend the organisms in a menstruum which will insure a water-clear suspension free from protein or pigment, and having a definite density of the organism. Since the penetrating power of the rays varies inversely as the turbidity of the bacterial suspension and the transparency of the suspending fluid, these factors must be subject to reasonably accurate control.

Experiment 1. The effect of sublethal doses of the ray upon meningococcus was studied and the first step was to determine the minimal lethal dose of ultraviolet light.

Technic: Meningococcus cultures representing the regular, irregular and para types were subcultured from an eighteen to twenty hour old sheep serum agar slant to glucose agar. After twenty hours incubation these were again transferred to glucose agar and the growth treated on the next day as follows. Standard suspensions were prepared in physiological salt solution to contain 2000 million cocci per cubic centimeter. These clear suspensions in amounts of 15 to 20 cc. were placed in sterile quartz Erlenmeyer flasks of 50 cc. capacity and exposed to ultra-violet rays for varying periods after which the flask was well shaken and 0.5 cc. of the suspension was withdrawn and pipetted on to sheep serum glucose agar. These subcultures were incubated for twenty-four hours and negative tübes were re-incubated for an additional day in order to determine possible attenuating action which might result from the different exposures. An "Alpine Sun Lamp," manufactured by the Hanovia Chemical Company was used with a current of 110 volts, 3 amperes, at a distance of 36 inches. The temperature of the flask when exposed directly to the mercury arc did not exceed 38°C. at any time.

Minimal lethal dose of ultraviolet ray on types of meningococcus

	TIME OF EXPOSURE (MINUTES)																
Culture..	½	1	1½	2	2½	3	3½	4	4½	5	5½	6	6½	7	7½	8	10
Regular..	++	++	++	++	++	++	++	++	++	++	++	++	+	+	–	–	–
Control..	Still fully viable after one hour																
Irregular.....	++	++	++	++	++	++	++	++	++	++	++	++	++	++	+	+	+
Control..	Still fully viable after one hour																
Para.....	++	++	++	++	++	++	++	++	++	++	++	+	+	–	–	–	–
Control..	Still fully viable after one hour																

Growth after twenty-four hours incubation indicated by ++. Negative growth after twenty-four hours but positive after forty-eight indicated by +. No growth indicated by –. Repeated three times with approximately same results.

In order of their susceptibility to the ultraviolet ray a parameningococcus comes first, followed by the regular and next by the irregular type. This, in general, appears to be in keeping with the resistance of each of these strains to environmental conditions as observed in the laboratory.

Effect of ultraviolet ray on agglutinogenic properties of meningococcus

Any change effected in the bacterial protoplasm by the physicochemical action of the ray should be coincident with a modification of a salient immunologic property. A monovalent serum prepared in the rabbit by means of a single type of meningococcus is highly specific, especially so when a rapid method of immunization is used. The studies of Gordon (16) and other workers have established this point and our own experience has not been otherwise. An irregular type serum may agglutinate to a slight degree a regular or a parameningococcus, but the reaction is never marked. On the other hand, a regular meningococcus produces no agglutinin for the para type—at least not above 1—25 in our observations—and slight agglutinins now and then for an irregular strain. The para type is highly specific, possessing slight group agglutinins for the irregular strains at times.

The object of the succeeding experiments was two-fold: first, to show, if possible, that the meningococcus antigen is complex and contains elements common to numerous types; secondly, to demonstrate the physicochemical influence of ultraviolet rays on a bacterial cell. This action should manifest itself either by a rearrangement of the internal structure of the organism with an alteration of its normal serologic reactions, or by bringing into prominence the group-radicals already present within the cell. The organism might then be shown to possess a wider range of agglutinating properties.

A regular no. 4, irregular no. 30 and a parameningococcus no. 60 were exposed to the action of ultraviolet rays for varying periods which corresponded to subminimal lethal doses determined previously. Duplicate sera were prepared in rabbits with each of these treated strains, a modified rapid method of immunization being used in which two daily injections were given followed by a rest period of six days after which the final injection was given. The amounts of culture used were respectively 1/100, 1/50 and one-quarter of an eighteen to twenty hour growth prepared as described in the technic for ultraviolet treatment. The animals were bled from the ear six days after the last injection and agglutination series set up against the untreated, living homologous and heterologous cultures. All agglutination tests were made at 56° C., and the results were read after eighteen to twenty hours incubation in the incubator.

Experiment 2. Regular meningococcus No. 4 was exposed to the ultraviolet ray for 1, 2, 3, 4, 5 and 6 minutes, respectively, and sera for each of these modified cultures were prepared as described above. The animals showed no ill effects from the injections.

SERUM	ULTRAVIOLET EXPOSURE		AGGLUTINATING RANGE							
			1-100	1-200	1-400	1-500	1-800	1-1000	1-1200	Control
	minutes									
176	1	4	+	+	±	±	−	−	−	−
		30	+	+	−	−	−	−	−	−
		60	+	+	+	+	±	−	−	−
177	2	4	+	+	±	±	±	−	−	−
		30	++	++	+	±	−	−	−	−
		60	+	+	+	−	−	−	−	−
178	3	4	++	++	+	±	−	−	−	−
		30	++	++	±	±	−	−	−	−
		60	+	+	±	−	−	−	−	−
179	4	4	++	++	++	++	+	±	±	−
		30	++	++	++	+	+	±	−	−
		60	+	±	±	−	−	−	−	−
180	5	4	+	+	±	±	±	−	−	−
		30	++	++	+	+	±	−	−	−
		60	++	+	+	+	±	−	−	−
181	6	4	+	+	+	±	−	−	−	−
		30	++	++	+	+	−	−	−	−
		60	±	±	−	−	−	−	−	−

In all protocols the following symbols are used to denote degree of agglutination. ++, complete, with clear supernatant fluid; +, sediment with slightly cloudy supernatant fluid; ±, flocculi in fine suspension; −, negative.

The conclusions to be drawn from the experiment are that exposures of one to five minutes to the action of ultraviolet light under certain conditions renders the antigen more inclusive; also, that a toxic organism, such as the regular meningococcus which was used in the experiment, may become less toxic by the treatment and may be tolerated in much larger doses on the second day without giving rise to severe reactions or death. The serum produced with such altered cultures agglutinates heterologous strains equally as well as or better than its specific antigen, an indication that a regular type meningococcus contains antigenic elements common to the irregular and para types.

Experiment 3. Irregular meningococcus No. 30 was exposed to the ultraviolet ray for 2, 4, and 10 minutes. Reference to the first table will show that distinct inhibition occurred with an exposure lasting ten minutes.

SERUM	ULTRAVIOLET EXPOSURE		AGGLUTINATING RANGE						
			1-100	1-200	1-400	1-500	1-800	1-1000	Control
	minutes								
182	2	30	++	++	++	+	−	−	−
		4	++	++	+	+	−	−	−
		60	++	++	+	+	±	±	−
183	4	30	++	+	−	−	−	−	−
		4	±	−	−	−	−	−	−
		60	++	++	+	±	−	−	−
186	10	30	++	++	+	+	±	−	−
		4	+	±	−	−	−	−	−
		60	++	++	++	++	+	−	−

All three sera showed marked agglutinating properties for the heterologous strains. The parameningococcus no. 60 was agglutinated in all cases to a greater degree than the specific antigen no. 30. Exposure to the rays brings out pronounced agglutinogens for the regular and para types.

Experiment 4. Parameningococcus no. 60 was exposed to ultraviolet rays for periods varying from one-half to six minutes.

SERUM	ULTRAVIOLET EXPOSURE		AGGLUTINATING RANGE								
			1-100	1-200	1-400	1-500	1-800	1-1000	1-1200	1-1500	Control
	minutes										
187	½	60	++	++	++	+	+	±	–	–	–
		4	–	–	–	–	–	–	–	–	–
		30	+	±	–	–	–	–	–	–	–
188	1½	60	++	++	++	±	±	–	–	–	–
		4	–	–	–	–	–	–	–	–	–
		30	+	±	–	–	–	–	–	–	–
190	4½	60	++	++	++	++	++	+	+	+	–
		4	±	–	–	–	–	–	–	–	–
		30	+	+	±	–	–	–	–	–	–
191	5½	60	++	++	++	++	+	+	+	+	–
		4	–	–	–	–	–	–	–	–	–
		30	++	+	±	–	–	–	–	–	–
192	6	60	++	++	++	++	+	+	+	+	–
		4	±	–	–	–	–	–	–	–	–
		30	++	+	+	±	–	–	–	–	–

The parameningococcus is not altered agglutinogenically by the ultraviolet ray with exposures ranging from one-half to six minutes. A serum prepared with such treated cultures is highly specific and indicates that the parameningococcus is a distinct type the serum of which rarely, if ever, possesses group agglutinins for the regular strain. However, it does include some agglutinin for the irregular type which is evidently more closely related to the parameningococcus. By the same token, a parameningococcus no. 60 possesses no agglutinogen radicals common to a regular meningococcus, whereas the regular type includes within its protein molecule the elements of the parameningococcus. Another point brought out by this experiment is the possibility of developing in rabbits sera of high titer by so few as three injections. This is especially marked in the last series where parameningococcus was studied. Exposure of this organism to the rays for four and one-half to six minutes appears to have made the antigens especially agglutinogenic for the homologous strain.

The effect of exposing a regular type of meningococcus to ultraviolet light was next studied in the monkey instead of the rabbit. The results were even more striking. A typical experiment is given.

Experiment 5. June 17, 1918. Macacus rhesus. Regular meningococcus no. 4 was grown for two generations on glucose agar, then exposed to ultraviolet radiation for five minutes. The technic of immunization and serum study were the same as those described previously. One-fourth culture of an eighteen to twenty hour growth injected intravenously. June 18, one-half of a similar culture injected intravenously. June 25, an entire culture of the same strain treated as before was injected intravenously. On July 1, the animal was bled from median vein. The agglutination test was set up on the same day.

CULTURE	AGGLUTINATING RANGE						
	1-100	1-200	1-400	1-500	1-800	1-1000	Control
4	+	−	−	−	−	−	−
30	+	+	±	±	−	−	−
60	++	++	++	+	±	−	−

In summarizing these experiments the conclusion may be drawn that exposure to the action of ultraviolet rays results possibly in a definite protoplasmic modification of microörganisms. This change may be shown in part by immune properties which become apparent in sera obtained by the injection of such altered bacteria. The action of ultraviolet radiation appears to be a distinct principle unrelated to the influence of heat upon the bacterial protoplasm, since a physical change from this source has been ruled out by careful control of temperature.

The nature of the bacterial modification induced by ultraviolet rays

The next series of experiments was designed to illustrate the nature of the change induced by treating meningococci with the ultraviolet ray. With a definite exposure it was possible to repeat regularly the antigenic modification observed in the first experiments. Therefore such intervals were selected as served to bring about the most inclusive range of agglutination. The agglutinins were studied by means of absorption tests.

Technic: Suspensions of meningococci were prepared and treated exactly, as in Experiment 1. Rabbits were injected intravenously on two successive days with 1/100 and 1/50 of the treated culture, respectively, then given a rest period of six days, following which one-quarter of a treated culture was given. Seven days later the animals were bled from the ear, the serum was heated at 53°C. for fifteen minutes and agglutination tests were made on the following day. The method of Tulloch (17) for absorption of meningococcus agglutinins was not used since in that procedure the absorbing suspensions are too thin and are insufficient even when doubly exposed to an agglutinating serum. To insure satisfactory absorption, sera were twice absorbed according to the following procedure. The serum, diluted 1–50, was added in amounts of 5 cc. directly to glucose agar slants of eighteen-hour old cultures of the meningococcus, the suspensions being made up to a turbidity standard of about 8000 million per cubic centimeter. After thorough emulsification, the mixtures were placed in centrifuge tubes, incubated for two hours at 37°C., then stored in ice chest at 4 to 8°C. for sixteen hours. After centrifugation at high speed for twenty minutes, the clear supernatant serum was again treated as before. The second centrifugation was prolonged to twenty-five minutes at high speed in order to remove all possible clouding. Absorbed sera were then tested for agglutinins against the homologous types and against those which were found to be agglutinable as a result of the action of the ultraviolet rays on the given antigen. All series were set up with eighteen hour old cultures grown on glucose agar and suspended in physiological salt solution to contain 2000 million meningococci per cubic centimeter. Each serum was controlled with a non-absorbed specimen which was subjected in parallel to every detail of the experimental conditions imposed by the method. A few typical results have been selected from duplicate sets of experiments.

Experiment 6. Regular meningococcus no. 4 was exposed to ultraviolet rays at a distance of 36 inches for four minutes.

CULTURE	AGGLUTINATING RANGE								
	1-100	1-200	1-400	1-500	1-800	1-1000	1-1200	1-1500	Control
4	++	++	++	++	+	+	+	+	–
30	++	++	++	++	+	+	±	±	–
60	+	±	±	–	–	–	–	–	–
After absorption with parameningococcus no. 60									
4	+	+	+	+	+	±	±	–	–
30	+	+	+	±	–	–	–	–	–
60	–	–	–	–	–	–	–	–	–
After absorption with irregular no. 30									
4	+	+	+	+	+	±	±	–	–
30	–	–	–	–	–	–	–	–	–
60	+	–	–	–	–	–	–	–	–
Control serum (not absorbed)									
4	+	+	+	+	+	±	±	–	–
30	+	+	+	±	–	–	–	–	–
60	+	–	–	–	–	–	–	–	–

Experiment 7. Irregular meningococcus no. 30 was exposed to ultraviolet rays for two minutes at a distance of 36 inches.

CULTURE	AGGLUTINATING RANGE							
	1-100	1-200	1-400	1-500	1-800	1-1000	1-1200	Control
4	++	+	+	+	±	–	–	–
30	++	++	++	++	++	+	±	–
60	++	++	++	++	+	+	–	–
After absorption with regular no. 4								
4	–	–	–	–	–	–	–	–
30	+	+	+	+	±	–	–	–
60	+	+	+	+	±	±	–	–
After absorption with para no. 60								
4	+	+	±	±	±	±	–	–
30	+	+	±	±	±	±	–	–
60	–	–	–	–	–	–	–	–
Control serum (not absorbed)								
4	+	+	+	±	±	–	–	–
30	+	+	+	+	±	±	–	–
60	+	+	+	+	±	±	–	–

Agglutinins newly developed in consequence of treating meningococci with ultraviolet light could be removed by a method of absorption, after which the sera so tested were still capable of agglutinating the other types with no appreciable diminution in antibody content. The results attest to some profound change in the bacterial cell.

The presence of group-agglutinogens in single bacterial types

If the theory be correct that micro-organisms contain radicals common to related groups of bacteria, then it should be possible to show that certain serologic manifestations developed artificially in a type organism are common to the untreated specimen. Accordingly, representative type meningococci were utilized in the following experiments as the homologous absorbing agents of different sera. These sera were prepared by injections with the same strains exposed to ultraviolet light and at the time the absorption tests were made they were one month old. The technic of absorption was the same as that previously outlined. Protocols representative of duplicated experiments are given. The series included the results obtained with different degrees of exposure to the rays, but these are omitted for the sake of brevity.

Experiment 8. Sera obtained from rabbits by injection with regular meningococcus no. 4 which was treated with ultraviolet rays, were absorbed with untreated meningococcus no. 4.

CULTURE	AGGLUTINATING RANGE AFTER ABSORPTION							
	1-100	1-200	1-400	1-500	1-800	1-1000	1-1200	Control
4	−	−	−	−	−	−	−	−
30	+	+	+	±	−	−	−	−
60	+	+	±	±	−	−	−	−
Control serum (not absorbed)								
4	++	++	++	++	+	+	±	−
30	++	++	+	±	−	−	−	−
60	+	+	±	±	−	−	−	−

Experiment 9. Sera similarly produced by systematic treatment with ray-exposed irregular meningococcus no. 30 were absorbed with the corresponding untreated strain.

CULTURE	AGGLUTINATING RANGE AFTER ABSORPTION							
	1-100	1-200	1-400	1-500	1-800	1-1000	1-1200	Control
30	−	−	−	−	−	−	−	−
4	+	±	−	−	−	−	−	−
60	++	++	+	±	±	±	−	−
Control serum (not absorbed)								
30	++	++	+	+	±	−	−	−
4	++	++	+	±	±	−	−	−
60	++	++	+	+	+	±	−	−

When sera containing newly formed agglutinins are absorbed with homologous types of meningococcus, the group-agglutinins are materially reduced for those organisms which appear to have antigenic radicals locked up within the major agglutinogenic complex. The relationship of some of the types may thus be shown. A regular type meningococcus contains antigenic elements common to the other groups. An irregular strain such as no. 30 appears to have in its make-up radicals largely of a regular type such as the no. 4 and to a slight degree those of a para strain represented by the no. 60. These results are instructive if compared with the behavior of sera which have been absorbed with organisms known to contain normal group-agglutinogens. As is well known, the secondary or minor agglutinins cannot be detected after the serum has been absorbed with the organism which has been used for immunization.

To carry this conception a step farther it is important to show if possible, that the immune properties developed in a micro-organism truly represent changes in the bacterial complex. If the theory be correct, we should be able to establish the relation between newly induced immune reactions and those present in the inciting agent. To test this point, the following experiments were devised.

Experiment 10. Sera were prepared in rabbits by injections of regular meningococcus no. 4 which was subjected to various exposures of ultraviolet light. These sera were then absorbed with the same strain of meningococcus modified by identical treatment. The results are summarized from a number of tests.

CULTURE	AGGLUTINATING RANGE AFTER ABSORPTION WITH REGULAR NO. 4						
	1-100	1-200	1-400	1-500	1-800	1-1000	Control
4	±	–	–	–	–	–	–
30	±	–	–	–	–	–	–
60	±	–	–	–	–	–	–
Control serum (not absorbed)							
4	++	++	+	+	±	–	–
30	+	+	±	±	±	–	–
60	+	+	±	–	–	–	–

Experiment 11. Sera were prepared as in the preceding experiment, with the use of an irregular meningococcus no. 30 treated with ultraviolet light. Absorptions were made with the same strain similarly treated.

CULTURE	AGGLUTINATING RANGE AFTER ABSORPTION WITH IRREGULAR NO. 30						
	1-100	1-200	1-400	1-500	1-800	1-1000	Control
30	–	–	–	–	–	–	–
4	–	–	–	–	–	–	–
60	±	–	–	–	–	–	–
Control serum (not absorbed)							
30	++	++	+	±	±	±	
4	++	+	±	–	–	–	
60	++	++	++	+	+	±	

The results are quite decisive. Preceding experiments have shown that newly formed agglutinins are specific for homologous or heterologous antigens possessing corresponding agglutinogenic radicals. The same tests when applied to an antigen so modified as to show its group affinities, establish the fact that the serum reactions may be traced to profound changes in the bacterial protoplasm, as evidenced by the complete disappearance of newly developed agglutinins for the heterologous strains.

DISCUSSION

The experiments recorded in this paper establish several points which bear directly upon the problem of immunity and group-relationships of bacteria. In the first place it has been shown that ultraviolet light is capable of modifying bacterial antigens in such a manner as to improve their immunizing properties. Secondly, their serologic manifestations are rendered more inclusive by this treatment. This method of study followed in analyzing the reactions of supposedly unique types of meningococcus has indicated that certain representatives of the species are composite antigens which contain cell radicals common to other members of the group. The relationships which may be brought out by a suitable absorption technic appear to be definitely correlated with profound changes exerted upon the bacterial cell by a physicochemical agent. These results are contrary to those of Gordon (18) whose "superimposition" tests argue for specifically distinct types.

The mechanism by which ultraviolet rays act upon bacterial protoplasm is not clear. The general impression gained from work done on this subject is that the rays coagulate the cell proteins. Those who have studied the problem, however, have limited their observations to the bactericidal action of the rays and have concluded that death of the organism results from coagulation of the protein. This is undoubtedly true for the method of experimentation followed by these workers since they failed to take into account the action of heat and therefore were not dealing with the effect of ultraviolet rays. By removing the factor of heat and working with ray exposures which are sublethal, one is able to arrive at a different conclusion. In this connection it is of interest to remark the close analogy which exists between the action of heat and some physicochemical agent in bringing about marked alteration of antigenic properties.

That agglutinogenic properties may be influenced by heat has been shown by Joos (19). A serum produced with heated bacteria agglutinates both heated and unheated organisms, whereas a serum obtained by injection of unheated bacteria agglutinates heated organisms less highly. Similar results have been obtained by Kraus and Joachim (20) and Scheller (21). Of especial importance is Scheller's observation that heated bacilli (typhoid) absorb agglutinins out of the sera more actively than do the unheated and that the highest agglutination titers can be obtained by injecting heated bacteria. The experiments with ultraviolet rays seem to embody some such change which is analogous to the alteration of bacterial cell bodies and, furthermore, they offer a method which leaves the bacteria intact and thus capable of exerting their maximum immunizing activities.

For want of a better term the idea of "molecular configuration" is suggested for the internal structure of bacteria and it is supposed that the characteristics of organisms are distributed uniformly throughout the cell. A physicochemical influence, such as ultraviolet light, acting upon the living cell, changes its internal structure by modifying all of the molecules which define the organism. The result is either a new combination of the elements already present, or a qualitative change exerted uniformly throughout the cell substance. The new characters which are so imparted must not be taken to imply mutation or a transformation of one type of organism into another.

Another point which might be discussed is the possible effect which sublethal doses of ultraviolet light may have upon the least resistant organisms. Assuming that these were killed off by the treatment, it would still be impossible to accept this in explanation for the altered immunity response to bacteria so exposed, since a reduction in numbers can have no influence on specificity to the extent observed in these experiments.

A consideration of the physiologic action of ultraviolet light is linked with the question of enzymes (22). Since these are concerned in the processes which we are able to study *in vitro* there are reasons for believing that changes effected by physicochemical agents may be correlated with alterations of such enzymes or with an activation of precursory enzymes in the cell. That the ultraviolet ray may have a marked effect on tryptic and diastatic ferments has been noted by some investigators, among whom may be cited Hertel (23). The method by which the rays are able to change the action of enzymes is not explained easily. We believe that we have given experimental evidence that some such change may be responsible for unusual serologic reactions.

The practical application of these findings is suggestive. In view of the alterations which ultraviolet rays can produce in micro-organisms without impairing their antigenic value, new fields are opened for the investigation of bacteria which have thus far proved refractory as antigens. The advantage of employing living, yet innocuous organisms is obvious. In a further communication we shall discuss, from this viewpoint, some experimental work with *Pneumococcus*. In this connection the effect of ultraviolet light on *B. tuberculosis* is of interest. It has been found, for example, that after exposure to these rays the organism loses its acid-fast properties (24). That the direct action on bacteria is of a comprehensive nature follows also from the loss of Gram-staining characters (25).

There are further corollaries to be deduced from the experiments. It should be possible to develop a degree of immunity in animals by the injection of organisms which for each dose have been subjected to a correspondingly shorter exposure to ultraviolet rays. This simulates, in a measure, the cycle through which an infective agent passes in the animal body in which a gradual and a correspondingly diminished attenuating influence operates prior to the overwhelming of the body by virulent organisms.

From the nature of group antigens it appears that a few strains may be used for vaccination against a large heterogeneous group of bacteria. We have here what seems to be a manifestation of a single protein (antigenic) structure which gives to related organisms the properties whereby they may be classified within a certain group. Bearing this in mind, the explanation for much of the success with non-specific vaccine and protein therapy becomes relatively simplified.

SUMMARY AND CONCLUSIONS

Types of meningococci have been subjected to ultraviolet treatment and changes in antigenic properties observed. By a technic of agglutinin absorption it has been shown that such modifications are attributable to the physicochemical action of the rays.

Carefully regulated exposures which exclude the influence of heat on the bacterial proteins exert a definite action on the cells. Such treatment appears to favor agglutinin response and to diminish the toxic effects of certain organisms.

The group relationships of bacterial types are brought out quite clearly when under the influence of a physicochemical agent which "unlocks" or alters antigenic properties within the cell.

The action of ultraviolet rays on micro-organisms as observed in these experiments suggests a method for building up an immunity in animals by injections of bacteria exposed to the rays for constantly diminishing periods of time. The importance of working below the lethal dose of ultraviolet rays is obvious.

Certain types of bacteria while supposedly containing only a major antigenic structure actually contain minor antigens also.

There is evidence that a few strains, or probably a single strain of bacteria may suffice for immunizing against a heterogeneous group of organisms.

The experiments suggest that a single protein (antigenic) structure represents the element common to groups of biologically related organisms.

REFERENCES

(1) **Duclaux**: Comptes rendus de l'Academie de Sciences, 1885, **100**, 119.
(2) **Arloing**: Comptes rendus de l'Academie de Sciences, 1885, **100**, 378.
(3) **Koch**: Tenth International Med. Congress, Berlin, 1890.
(4) **Mignesco**: Archiv für Hygiene, 1896, **25**, 361.
(5) **Ward**: Proceedings Royal Society, 1893, **52**, 393.
(6) **Buchner**: Archiv für Hygiene, 1893, **17**, 179.
(7) **Cernovodeanu and Henri**: Comptes rendus des Séances de l'Academie des Sciences, 1910, **150**, 52.
(8) **Jodlbauer and Tappeiner**: Münch. Med. Wochenschr., 1904, p. 737; ibid., p. 1096, p. 1139.

(9) TAPPEINER: Münch. Med. Wochenschr., 1900, p. 5; 1901, p. 1810; 1904, p. 714.

(10) FINSSEN: Chemiche Strahlen des Lichtes für Medicin und Biologie, Leipzig, 1899.

(11) CERNOVODEANU AND HENRI: Loc. cit.

(12) BURGE: Am. Jour. Physiol., 1915, **38**, 399; 1917, **43**, 429.

(13) BUCHNER: Loc. cit.

(14) DESFOSSES: La Presse Medicale, 1913, p. 453.

(15) NAGIER: La Presse Medicale, 1913, p. 612.

(16) GORDON: Special Report no. 3, Eng. Med., Research Committee, London, 1917, p. 27.

(17) TULLOCH: Jour. Roy. Army Med. Corps, 1917, **29**, 66.

(18) GORDON: Loc. cit.

(19) JOOS: Centralblatt für Bakteriologie, 1903, **33**, 762.

(20) KRAUS AND JOACHIM: Ibid., 1904, **36**, 662.

(21) SCHELLER: Ibid., 1904, **36**, 427, 694; 1905, **38**, 100.

(22) RICHARDS AND WOODWARD: Am. Jour. of Roentgenology, 1917, **4**, 564.

(23) HERTEL: Zeitschr. für Allgemeine Physiologie, Verworn, 1905, **5**, 95; 1904, **4**, 1.

(24) CERNOVODEANU AND HENRI: Loc. cit.

(25) CERNOVODEANU AND HENRI: Loc. cit.

HYPERSENSITIVENESS: ANAPHYLAXIS AND ALLERGY

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The subject of hypersensitiveness dates from the earliest observations upon human idiosyncrasy when it was noted that certain persons reacted, with peculiar symptoms, to contact with substances, such as foods, drugs, animal emanations and pollens, which to the great majority of individuals were entirely innocuous. Considerable added interest was infused into the study of this natural hypersensitiveness of human beings by the observations of Richet, Arthus, Theobald Smith and many others upon the experimental hypersensitiveness of lower animals. One of the outstanding results of this stimulated interest was the generally accepted inclusion of all of the known phenomena of peculiar or unusual physiological reactivity under one heading—that of anaphylaxis.

In the designation of such phenomena in one group the quality of peculiar reaction has generally been the only one taken into account, notwithstanding obvious and often radical differences among some of them as to their nature and causation. At present no etiological subdivision of these various phenomena is recognized.

It is the purpose of this article briefly to present restrictive definitions of the terms in general use in the literature of the subject and to classify the known phenomena of peculiar physiological reaction in accordance with these definitions.[1]

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[1] A fuller exposition of this question is contained in the article on "Hypersensitiveness" in the forthcoming Practice of Medicine edited by Dr. Frederick Tice.

It may be that the term hypersensitiveness will always be needed for use in a general sense applicable to conditions of non-specific, exaggerated or unusual reactivity on the part of a living organism. Such conditions are illustrated in the exaggerated sensitiveness to light of the eye in conjunctivitis.

There are, however, two groups of the phenomena of peculiar, physiological reactivity, which, on account of definite constant features, can be separated from the others etiologically and these, though themselves of different etiology,[2] can be associated under the heading "Hypersensitiveness." These two groups have been designated with the familiar terms "Anaphylaxis" and "Allergy."

From the determining features of these two groups has been drawn the following definition of true hypersensitiveness; namely, a condition of specific or particular reactivity, with characteristic symptoms, to the administration of or contact with any substance in a quantity which to most of the individuals of the same species is innocuous.

This definition should be amplified by the following restrictive explanation:

1. The "characteristic symptoms" are generally different in the different animal species for the same group of substances.
2. They are uniform in any one species for various substances.
3. Where the exciting agent possesses a normal physiological action; for example, the drugs, the symptoms of this action are, with few exceptions, different from those of hypersensitiveness to that agent.[3]

By the terms of the foregoing definition the tuberculin reaction of Koch and the so-called toxin hypersensitiveness are excluded from the category of true hypersensitiveness; because:

1. The symptoms of the tuberculin reaction are the same in all animal species.

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[2] Etiology refers, here, to the origin of the underlying conditions, not to the exciting agents, which are often identical in both groups.

[3] All of these facts concerning the symptoms of true hypersensitiveness point to the conclusion that the phenomena under consideration are not dependent on any special property of the exciting agent, but solely upon a peculiar adjustment of the hypersensitive individual toward that agent.

2. The symptoms of toxin hypersensitiveness are *not* different from those of the normal physiological effect of the agent.

The phenomena of true hypersensitiveness are classified by the writer as those of anaphylaxis and those of allergy and, as it will presently appear, these terms are not used as synonyms but as mutually exclusive terms.

Anaphylaxis is a state of true hypersensitiveness that is due to the presence, in certain tissues, of specific antibodies. The symptoms of anaphylaxis are caused by the meeting of these antibodies with the respective antigen in those tissues. This definition may be elucidated by the following facts:

1. The state of anaphylaxis does not occur spontaneously but as a result of an immunological procedure (active or passive). It is experimental. The exciting agents are always antigens capable of inducing the formation of precipitating antibodies.

2. Anaphylactic hypersentiveness is not inheritable; that is, it is not transmissible to the offspring from the male parent. The hypersensitiveness that is sometimes transmitted from the mother to the offspring is always identical in both with respect to the exciting agent. In such a case the transmission of the hypersensitiveness does not occur by inheritance but by a transferal of specific antibodies from the blood of the mother to that of the offspring through the placenta.

3. An animal that is anaphylactic can always be rendered specifically and completely insensitive by a suitable (gradual) neutralization of the antibodies which are responsible for the hypersensitiveness. This procedure is known as "desensitization."

If we examine the facts concerning the causation and symptoms of human hypersensitiveness or allergy, we find full conformity with the definition of true hypersensitiveness.

1. The condition is highly specific in the sense that it is exibited in about half of the hypersensitive individuals to only one substance (Cooke and Vander Veer), sometimes indeed, to only one chemical group or element.

2. The symptoms are generally different from those of hypersensitiveness in other animal species (the coryza, the gastrointestinal symptom complex, eruptions).

3. The symptoms are the same for a great variety of different substances.

In these three characteristics the hypersensitiveness of allergy resembles that of anaphylaxis. In all other respects, however, the two conditions are diametrically dissimilar. This dissimilarity is evident in the following comparison:

1. The exciting agent in anaphylaxis is always an antigenic substance; that in allergy is often non-antigenic and cannot, therefore, induce a condition of anaphylaxis.

2. The hypersensitiveness of anaphylaxis is always induced by previous introduction of an undigested antigenic substance into the body of an experimental animal. Such a procedure has not been shown to induce allergic hypersensitiveness in human beings and the weight of evidence is overwhelmingly against such an assumption. Allergy is often exhibited immediately upon the first contact with the exciting agent.

3. The hypersensitiveness of anaphylaxis is not a heritable condition. That of allergy has been proven to be inherited, and when the mother is affected the hypersensitiveness of the offspring seems to be more often exhibited to a different substance than the one that affects the mother (Cooke and Vander Veer).

4. The phenomenon of desensitization, which never fails in anaphylaxis, is entirely wanting in allergy. It is true that a certain degree of lessened sensitiveness to *natural contact* with the exciting agent is often attained in allergy, which may be termed, *clinical insensitiveness.* In all such cases, however, the suitable administration of the exciting agent, by intracutaneous or subcutaneous injection, will demonstrate the persistence of the hypersensitiveness (Cooke).

The differences between these two conditions, which have just been mentioned, may be summarized as follows:

Anaphylaxis is an experimental, or induced, non-heritable hypersensitiveness due to the presence of specific antibodies in certain tissues.

Allergy is a natural inherited condition of hypersensitiveness, which affects only human beings and is not dependent in any way on immunological antibodies.

It is clear from this comparison that the true hypersensitiveness of human beings, which we have designated as "allergy," must rest on an etiological basis that is quite different from that of anaphylaxis.

The question naturally presents itself, cannot the hypersensitiveness of anaphylaxis be induced in human beings as well as in lower animals?

It may be pointed out, here, that not all lower animals have been rendered anaphylactic.[4] The negative experiments of Yamanouchi and also by Uhlenhuth and Haendel with apes have been confirmed with a much larger number of animals by Auer in unpublished observations.[5]

For the certain recognition of anaphylaxis in human beings it would be necessary that peculiar and characteristic symptoms develop *only* after a *reinjection* of an antigenic substance. If the same symptoms are seen to be produced often by a primary injection of the antigenic substance or, indeed, more frequently after a primary injection than after a reinjection, then such evidence could hardly be used as indicating the existence of a condition of anaphylaxis. The mere absence of effect at the first injection by no means proves that the effect of the reinjection is due to the immunological mechanism. The difficulty of recognizing the hypothetical anaphylactic symptoms following the reinjection of a true antigen must increase if, as is actually the case, the symptoms observed under such circumstances are never different from those of allergy.

It becomes necessary at this point to identify the allergic symptoms; that is, to determine what symptoms in human beings may be considered to be allergic.

On the one hand allergic conditions must conform to the general criteria of hypersensitiveness and, on the other hand, they must present some feature which will exclude them from the category of anaphylaxis.

Under the definition of hypersensitiveness laid down above, allergic symptoms may be identified by their specificity, their unusual occurrence, their uniformity with different exciting agents and their difference from the normal physiological effect of the agent.

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[4] Only the usual criteria of outward symptoms and death are considered here.

[5] Personal communication.

It is a striking feature of allergy that these requirements are met by several symptoms[6] or symptom-complexes. Thus, coryza bronchial asthma, gastro-intestinal disturbance, multiform cutaneous eruptions and sudden death following dyspnoea and respiratory failure, are all seen, in some individuals, to result from natural contact with or the administration of substances that are, to most individuals, entirely innocuous.

All of these symptoms are found to occur under circumstances that exclude the possibility of an anaphylactic etiology. Such circumstances are:

1. The non-antigenic nature of the exciting agent as in drug allergy ("idiosyncrasy").
2. The development of the symptoms at the first contact with the agent, as in many cases of "serum sickness" without incubation period.
3. The demonstration of the factor of heredity in the causation of the symptoms.

Having identified the various symptoms of allergy we may turn again to the question whether anaphylactic hypersensitiveness does actually occur in man. We can consider this question most conveniently by examining the effects in human beings of the injection of therapeutic sera; because the material injected is a known and commonly employed anaphylactogen and because the conditions under which such sera are administered often approximate those of the anaphylaxis experiment.

The injection of therapeutic serum, which is practically always derived from the horse, is often followed by a reaction known as "serum disease." The symptoms of this condition are those of allergy and they include the multiform eruptions, fever, edema, joint pains and sudden death. All of these symptoms and some other minor manifestations have been seen to follow a *primary* injection of the serum; that is, under a circumstance which, alone, would seem sufficient to rule out an anaphylactic (antibody-antigen) mechanism. Indeed the fact should be emphasized that the vast majority of the instances of serum disease occur after a primary injection.

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[6] It is possible that there are other clinical manifestations of allergy that have not yet been recognized as such.

The character of the symptoms in any particular individual is determined in part by the mode of contact with the exciting agent. For example, the exciting agent of animal emanations if inhaled may cause coryza; if injected under the skin it may cause sudden death.

Additional evidence of the non-anaphylactic nature of the "serum disease" that results from a primary injection of serum is found in the constant absence of the phenomenon of desensitization in that condition.

It is important, here, to note the effect of a reinjection of serum upon the course of serum disease in individuals that presented that condition at the first injection. Under these circumstances the symptoms following the reinjection *do not differ in character* from those that were produced by the first administration of the serum. However, there is no doubt that the period of incubation is generally shorter at the reinjection and that the severity of the symptoms is often increased. It must be emphasized that no evidence exists to indicate that this difference between the reactions occuring at a first injection and at a reinjection depends on any immunological influence.

There are left to be considered the relatively few instances in which, as in the anaphylaxis experiment in the lower animals, symptoms resulted in human beings from the reinjection but not from the first injection. These circumstances are analogous to those of active anaphylaxis and if allergy did not exist they could be accepted as representing anaphylaxis in man. However, in none of these instances have symptoms been noted which were characteristically different from those of allergy. They have been either the usual eruptive manifestations or collapse followed, in some cases, by death.

Hence, the only reason for suspecting these cases to be instances of anaphylaxis and not of allergy is the absence of symptoms at the first administration of the serum, and this circumstance cannot be satisfactorily explained until the mechanism of allergy is known. Certainly, it is not sufficient basis for a separation of these instances from the category of allergy.

In this connection it is well to bear in mind the instances in which the symptoms of drug allergy were absent at the first administration of the drug but appeared after a repeated administration. Only very few such instances have been reported, probably because many of those who have observed them have overlooked their theoretical interest.

I am permitted by Dr. John A. Fordyce to refer, in advance of their publication, to some instances of this kind that have occurred in his experience. In as many as ten or twelve cases no symptoms were caused by the first four or five intravenous injections of salvarsan. The fifth or sixth injection was followed by allergic symptoms (eruption) which recurred at all subsequent administrations, although sometimes long intervals of time (one to three years) elapsed between successive injections. In a personal communication, Dr. Fordyce remarks:

> The amount of the drug which produces the relapsing eruption apparently has no special significance. The amount of drug injected after these intervals [one to three years] has usually been a minimum quantity, about 0.25 to 0.3 of a gram of salvarsan.

An exactly similar case has been observed in the dermatological clinic in Cornell University Medical College, which will be reported by Dr. Oscar L. Levin.

Thus, it is evident that the mere absence of symptoms upon a first injection of serum is not sufficient to indicate that the symptoms occurring upon subsequent injection are of anaphylactic origin; there was previous evidence, which is now confirmed by the more numerous observations of Fordyce and that of Levin, that this occurrence is a characteristic phenomenon of drug allergy—a condition obviously unrelated to anaphylaxis.

Even in the few cases, therefore, in which the possibility of the operation of an anaphylactic mechanism could be considered; that is, those in which symptoms developed only upon a reinjection of serum, there appears to be no good ground for looking upon these as anything else than less usual forms of allergy.

As is well known, the proof that the manifestations of anaphylaxis are due to an antibody-antigen reaction was provided in Otto's demonstration of passive sensitization.

The principle embodied in the experiment of passive sensitization has been applied in serum and food allergies and even in non-antigenic drug allergy and the ultimate purpose of such applications of the principle has been to prove the anaphylactic nature of those allergies. However, the purpose of such experiments has been nullified by a fallacy which seems to have been overlooked. The technic of passive sensitization can be used to detect the presence of precipitin in an individual's blood, but if the presence of precipitin has been demonstrated by this means it does not follow that the individual under examination is anaphylactic. For example: guinea-pigs have been passively sensitized with serum from an immunized monkey, yet in not a single instance in numerous experiments has the monkey itself been rendered anaphylactic.

In applying the technic of passive sensitization in the study of allergy, the investigators have transferred the serum of the human individual almost exclusively to the guinea-pig, in which, then sensitization to the exciting agent of the allergy was looked for. However, in one instance reported by Ramirez,[7] a quantity of blood (600 cc.) was transfused from an individual that was hypersensitive to horse dander to an anaemic patient who had previously exhibited no symptoms of allergy. Two weeks after the transfusion the recipient went for a carriage drive and was seized at once with an attack of asthma. The usual cutaneous test revealed in the patient a hypersensitiveness to horse dander. Unfortunately, it is not known whether the cutaneous hypersensitiveness existed previous to the transfusion or not.

This observation is unique in the records. If it should be found that under similar circumstances hypersensitiveness could be regularly or often transferred from one human individual to another, it would be necessary to revise the conception of allergy that is presented in the foregoing pages. For the present, it seems proper to look upon the occurrence reported by Ramirez as an accidental coincidence. Some support is given to this view by the fact, which appears in the paper of Ramirez, that the same donor had supplied a larger quantity of blood (800 cc.) to another recipient, who, however, did not develop hypersensitiveness to horse.

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[7] Journ. A. M. A., 1920, 73, 984.

The age of onset of the clinical manifestations of allergy is different in different individuals. Cooke and Vander Veer have shown that the age of onset depends entirely upon a hereditary influence. It seems more probable that the recipient observed by Ramirez had just reached the age of natural onset of the horse allergy when the transfusion was carried out, than that the transfusion itself was the cause of the allergy.

In view of the facts that form the basis of the foregoing discussion, it seems necessary to conclude: first, that if anaphylaxis does occur in man, it does so only very rarely and secondly, that there is no positive evidence that anaphylaxis occurs at all in human beings.

THE RELATION OF SPUTUM BACTERIA TO ASTHMA[1]

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It is now recognized that asthma is a symptom complex which depends on one or the other of two great groups of causes: either the cause is a foreign protein which exerts its influence from outside the body—"extrinsic"—or it produces its effect from some focus, usually of bacterial growth and action within the body —"intrinsic."

The treatment of this last group consists either in eradicating the focus by surgical means if possible or in the use of bacterial vaccines made preferably from the same strains which are causing the focus of infection or finally by surgery and vaccination together.

Inasmuch as the search for a "focus" is usually futile, it is assumed that a chronic infection of the bronchi is responsible for the asthma and vaccines are prepared from the sputum bacteria.

Treatment with such vaccines has been used with success by several workers. Montgomery and Sicard (1) isolated streptococci, either hemolytic or non-hemolytic from each sputum and claim that out of 16 cases treated with autogenous vaccines, 12 were cured, 3 improved and 1 unimproved. The work of Walker (2) is of greater interest: Out of 178 patients treated with vaccines, 28 (15.7 per cent) were found to be sensitive to bacteria and were treated accordingly with 75 per cent good results. Seventy-five other patients were treated only with the predominant organism with only 40 per cent good results.

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[1] Read at the meeting of the American Society for the Advancement of Clinical Investigation, May 3, 1920.

In an attempt to place the vaccine treatment of asthma on a more definite basis the following method of study was carried out in 40 cases. A smear from a suitable nugget of sputum obtained in a sterile vessel was made on a blood agar plate. Colonies from this first plate were then streaked in the sections of a second blood agar plate to insure purity and from these sections, tubes of dextrose broth were inoculated. The twenty-four hour growth in broth was then washed three times with saline containing 0.5 per cent carbolic acid, was finally suspended in the same and was killed by heating to 56°C. for one hour. The vaccines thus made were diluted so as to each have the same degree of cloudiness, as a suspension containing in 1 cc. 500,000,000 bacteria.

One hundred and twenty-nine organisms were isolated in this way. There were:

			per cent
77	Non-hemolytic streptococci		60
17	Hemolytic streptococci		13
17	Staphylococcus albus		13
7	Gram-negative cocci		6
5	Staphylococcus aureus	11 others	8
3	Pneumococci		
2	Gram-negative bacilli		
1	Diphtheroid		

Attention is called to the fact that of these organisms most numerous in the sputum of asthmatics, streptococci, chiefly non-hemolytic streptococci were found to make up nearly 75 per cent. Also that in the entire group, pneumococci were found only three times. That this last finding is dependent entirely on the technic used and probably does not represent the true condition, is shown by the work of Stillman (3) who in studying the organisms present in normal saliva, was able to recover pneumococci by mouse passage in 47 instances in none of which were these organisms recovered by direct sputum culture on blood agar plates—indeed pneumococci were recovered by the two methods at the same time in only 11 out of 116 specimens of sputum studied in this way.

With pure vaccines made from these organisms, intradermal tests were performed in the usual manner injecting about 0.02 cc. of the carbolized bacterial suspension. The tests were controlled by simultaneous injections of heterologous vaccines placed alternately with the autogenous. They were also controlled by injection into other patients.

The early reading of the tests was made in one-half hour and the late reading in twenty-four hours. Positive early tests were found to consist of an urticarial wheal surrounded by erythema much like the usual tests to pollens, but positive late tests were found to resemble an inflammation with its area of redness, swelling and slight tenderness. In the interpretation, the comparison of each test with its fellows was considered to be of far greater importance than any actual measurement of its size.

Skin tests with autogenous vaccines were done in this way in 39 patients; 25 of this group of 39 patients gave a positive test to one or more vaccines. There were 20 positive tests to an autogenous vaccine and 15 to a heterologous vaccine. In 17 additional patients, tests were done with only heterologous vaccines and 9 of these patients reacted positively. Thus in the whole group of 56 patients, 34 or 60.7 per cent gave a positive skin test. This figure includes those who gave only a slight test and is therefore higher than Walker's figure of 15.7 per cent positive. Attention is called to the fact that these tests were made by the intradermal method.

Of the 358 individual intradermal injections in the whole series of 56 patients, there were 70 (19.5 per cent) which were classed as positive. This 19.5 per cent includes 7.5 per cent which were early positives, 7.2 per cent which were late positives and in addition 4.8 per cent which were positive both early and late. Thus the occurrence of positive tests was by no means limited to autogenous vaccines, nor except in one case, were more tests obtained with one organism or group of organisms than another.

It is of interest to examine these tests with reference to the individual organism used. For this purpose only those organisms which were tested on 5 or more different persons, a total of only 32, are here included. However with these 32 organisms, a total of 283 tests were made—an average of 9 each.

Fourteen of these 32 organisms (almost half) gave no test at all and of the 4 organisms which gave more than 2 positives 1 was a hemolytic streptococcus with 3 positives, 2 were staphylococcus albus with 4 each and 1 was a gram negative bacillus which seemed to be irritative because 10 positives were obtained in 18 tests.

Treatment of these patients was carried out with small doses of pure vaccines given at seven-day intervals, each succeeding dose being regulated according to the amount of local reaction from the previous dose. If this local reaction was large, the next dose was made less. Any successful results appeared usually after 5 or 6 treatments, although in many of the unsuccessful cases as many as 12 or 15 treatments were given. The word "successful" is here used to denote a definite improvement in the subjective and objective signs and symptoms. It includes, however, only 2 instances of a virtual cure.

It was found that treatment was successful in fairly close accordance with the presence of a positive skin test. Ten patients who gave a positive test to their own organism were treated with this organism, all of them with success. Six patients were treated with a heterologous organism, 4 of them with success. However, in the face of a negative test 10 other patients were treated, 8 of them with their own organism and 2 of them with a heterologous organism, but with no success whatever. The distinctions in prognosis between an early and late test were found to be unimportant. Good results in treatment were obtained after both; in fact of the 2 patients who were "cured" (in quotation marks) 1 gave an early while the other gave a late test.

The organisms used in the treatment of the 14 successful cases included: non-hemolytic streptococcus 6, hemolytic streptococcus 3, staphylococcus albus 3, staphylococcus aureus 2, gram negative bacillus 1, Gram negative coccus 1; while those used in the 10 unsuccessful cases included: non-hemolytic streptococcus 7, staphylococcus albus 2, Gram negative coccus 1. In the 2 cases giving a positive skin test but treated unsuccessfully a non-hemolytic streptococcus was used in each.

The permanency of the favorable results obtained is yet to be discovered but most of these patients have retained at least during the seven months of observation the result which followed the treatment as given.

Aside from being an index as to the probable outcome of treatment, it was hoped that the skin test might become another method of differentiating various sub-groups of organisms and as a matter of fact, in the skin of the same patient, definite differences of reactions were obtained with different cultures of not only hemolytic and non-hemolytic streptococci but also of staphylococci. The data on this point is, however, far from complete.

Granted that the results outlined above are substantial and that the importance of a positive skin test as a prerequisite to successful treatment is not overestimated, we may discuss intrinsic asthma as follows:

We know that in horse asthma and ragweed pollen asthma, the symptoms depend upon an exquisite sensitiveness to the particular foreign protein. Inasmuch as circulating antibodies are not found, we assume that this condition of sensitiveness is cellular. The specific protein will produce a positive skin test and repeated injections will cause relief of symptoms. This treatment is specific.

In intrinsic asthma, vaccines likewise produce a positive skin test and as treatment with them is successful only in case the test is positive, their action is "specific." By analogy, therefore we may assume that asthma due to bacteria depends probably on a condition of specific cellular sensitiveness either to the bacteria themselves or to the products of their action in the organism.

REFERENCES.

(1) Montgomery and Sicard: Treatment of bronchial asthma by vaccination, with report of cases. Am. J. Med. Sci., 1917, **158**, 856.

(2) Walker, I. C.: Treatment of bronchial asthma with vaccines. Arch. Int. Med., 1919, **23**, 220.

(3) Stillman, E. G.: A contribution to the epidemiology of lobar pneumonia. Journ. Exp. Med., 1916, **24**, 651.

SOME OBSERVATIONS ON THE CONSTITUTION OF THE COMPLEMENTS OF DIFFERENT ANIMALS

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In the past a considerable amount of attention has been paid by serological workers to the complex characters of serum-complement and many attempts have been made to subject complement to more detailed biochemical analysis. Valuable information has thus been obtained regarding the biological action and properties of this important element of animal serum.

The study of complement has been principally based on its cytolytic effects towards red blood corpuscles sensitised with the homologous immune body, and the general tendency of research on this subject has been to elicit the complexity of constitution of complement and the numerous factors on which its action depends.

The fresh serum of the guinea-pig represents with ox corpuscles + rabbit versus ox immune body or cobra venom, one of the most active complements and it has therefore been commonly used for studies on complement action.

Attention was first drawn by Stephens (1) to the hemolytic effect of certain snake venoms along with fresh serum. In the case of guinea-pig's serum, which has a powerful activating effect, this characteristic property is annulled by heating the serum at 55°C. and corresponds as regards thermolability to the complementing action of serum with immune body. Observations made by Browning and Mackie (2) on the complementing action of serum with immune body in relation to its hemolytic effect with cobra venom showed that the venom-activating constituent of serum though similar in many characters to the complement which acted with immune body was not identical with the latter.

Ferrata (3) and later Brand (4) Liefman (5) Sachs (6) demonstrated that complement could be fractioned into two components neither of which exhibited any degree of complementing effect by itself, although together they reproduced the full hemolytic activity of the native serum. These two moieties were designated "end-piece" and "mid-piece" the latter combining directly with red blood corpuscles + immune body.

In Liefman's method carbon dioxide gas was passed through serum diluted with distilled water and this led to the precipitation of part of the globulins of the serum which represented the "mid-piece" while the "end-piece" consisted of the albumin and that portion of the globulin still remaining in solution. These fractions were carefully studied by Browning and Mackie in the case of guinea-pig's serum and it was found that the venom-activating constituent could also be fractioned into two similar components.

A "third component" of complement was described by Ritz (7) which had no end-piece or mid-piece properties and unlike these components was invariably stable at 57°C. This constituent was demonstrated by inactivating complement with venom and by the restoration of its activity on the addition of heated serum.

It was subsequently found (Browning and Mackie (8)) that complement could be fractioned into four different components by Liefman's method followed by precipitation of the proteins in different concentrations of ammonium sulphate. These four components were represented respectively by the albumin, pseudoglobulin from "end-piece," pseudoglobulin from "mid-piece" and euglobulin. None of these fractions corresponded to any of the previously described complement components "end-piece," "mid-piece" or "third component." These observations were carried out with guinea-pig's serum and the general results showed that the complement constituents were distributed over all the different proteins of the serum but appeared to be concentrated chiefly in the pseudoglobulin. Of the four components, three, including always the albumin, were generally necessary for full restoration of complement action and the albumin fraction appeared to represent an essential constituent of the complement.

While it is doubtful if albumin, pseudoglobulin and euglobulin separated by ammonium sulphate constitute homogeneous protein entities (Martin and Chick (9)), there is strong evidence that they represent different complement constituents, and Liefman's method certainly elicits a striking difference between the two moieties of the pseudoglobulin separated by carbon dioxide. With a view to throwing further light on the structure of complement and especially the venom-activating constituent of serum, further experiments have been carried out with the sera of certain other animals.

The technique followed was that originally described in the Journal of Pathology and Bacteriology and the Zeitschrift für Immunitätsforschung (8).

The hemolytic systems used were (1) ox red blood corpuscles + immune body (rabbit versus ox), and (2) ox red blood corpuscles + cobra venom. The sera of man, rabbit, and horse were selected for comparison with guinea-pig's complement.

With ox corpuscles + immune body rabbit versus ox the average minimal hemolytic dose of these sera as shown by Muir (11) are: Guinea-pig, 0.01 cc.; rabbit, 0.1 cc.; man 0.11 cc.; horse, ∞ cc.; for 1 cc. of a 5 per cent suspension of blood.

EXPERIMENTS WITH RABBIT'S SERUM

Various specimens of rabbit's serum were fractioned by Liefman's carbon dioxide method into "end-piece" and "mid-piece" and the two moieties were further subdivided by the ammonium sulphate method.

The different globulins were then tested separately and in certain combination as regards their complementing action with immune body.

Table 1 demonstrates the results of one of these experiments; the pseudoglobulin from end-piece showed distinct activity which was increased to the standard of the native serum by the addition of the pseudoglobulin from mid-piece; the latter by itself displayed no complementing properties. The addition of euglobulin to the mixture of pseudoglobulins did not further add to the hemolytic value. A mixture of the pseudoglobulin from end-piece and euglobulin also yielded a fully active complement but euglobulin along with pseudoglobulin from mid-piece was quite inactive.

Thus the complementing property of rabbit's serum is invariably resident in the globulins and distributed among them; but a mixture consisting of only two of these globulin fractions, provided they do not both belong to the mid-piece, is sufficient to reconstitute the complement. In the case of rabbit's serum also certain constituents may be considered as interchangeable. It is to be noted that there is an actual qualitative differentiation of the pseudoglobulin of end-piece and that contained in the mid-piece fraction. This was also noted in the case of guinea-pig's serum. In contrast with guinea-pig's serum however the albumin fraction does not appear to contain any complement constituents.

TABLE 1

RABBIT'S SERUM	LYSIS OF 0.5 CC. OX BLOOD SUSPENSION + 5 DOSES OF IMMUNE BODY				
	0.01 cc.	0.025 cc.	0.05 cc.	0.075 cc.	0.1 cc.
Native complement	tr.	dist.	c.	c.	c.
Albumin	0	0	0	0	0
Pseudoglobulin from end-piece	0	0	0	dist.	c.
Pseudoglobulin from mid-piece	0	0	0	0	0
Euglobulin	0	0	0	0	0
Pseudoglobulin from end-piece + pseudoglobulin from mid-piece	0	tr.	c.	c.	c.
Pseudoglobulin from end-piece + pseudoglobulin from mid-piece + euglobulin	0	dist.	c.	c.	c.
Pseudoglobulin from mid-piece + euglobulin	0	0	0	0	0
All four components	0	dist.	c.	c.	c.
Pseudoglobulin from end-piece + euglobulin	0	dist.	c.	c.	c.
Albumin + pseudoglobulin from mid-piece	0	0	0	0	0
Albumin + euglobulin	0	0	0	0	0

In this and in subsequent tables: tr. = trace; f.tr. = faint trace; dist. = distinct; mk. = marked; c. = complete; j.c. = just complete; al.c. = almost complete.

In general, rabbit's serum has no activating effect with cobra venom and ox's corpuscles, though with immune body it shows marked complementing action.

TABLE 2A

RABBIT'S SERUM	LYSIS OF 0.5 CC. OF 5 PER CENT SUSPENSION OX BLOOD + 0.0005 GRAM COBRA VENOM						
	0.04 cc.	0.1 cc.	0.16 cc.	0.2 cc.	0.24 cc.	0.3 cc.	0.36 cc.
Fresh serum...........	0	0	0	0	0	0	0
Globulin precipitate by $(NH_4)_2SO_4$...........	0	0	tr.	dist.	mk.	al.c.	c.
Albumin separated by $(NH_4)_2SO_4$...........	0	0	0	0	0	0	0
Globulin + Albumin: Globulin $(NH_4)_2SO_4$...	0.2 cc.	0.24 cc.	0.3 cc.	0.36 cc.			
	+	+	+	+			
Albumin $(NH_4)_2SO_4$...	0.05 cc.	0.06 cc.	0.075 cc.	0.09 cc.			
	0	0	0	0			

TABLE 2B

RABBIT'S SERUM	LYSIS OF 0.5 CC. OF 5 PER CENT OX BLOOD SUSPENSION + 5 DOSES IMMUNE BODY						
	0.01 cc.	0.025 cc.	0.05 cc.	0.075 cc.	0.1 cc.	0.2 cc.	0.3 cc.
Native serum....................	0	mk.	al.c.	c.	c.	c.	c.
Globulin $(NH_4)_2SO_4$ method.....		mk.	c.	c.	c.	c.	c.
Albumin $(NH_4)_2SO_4$ method.....	0	0	0	0	0	0	0
Globulin + albumin $(NH_4)_2SO_4$ method........................		mk.	c.	c.	c.	c.	c.

	LYSIS OF 0.5 CC. OF OX BLOOD SUSPENSION (NO IMMUNE BODY)
Globulin $(NH_4)_2SO_4$.............	0.3 cc. = no lysis
Native serum..................	0.3 cc. = no lysis

It was found however that the globulins separated by half saturation with ammonium sulphate showed distinct complementing action with cobra venom.

The albumin was inactive and also inhibited the action of the globulin even in doses corresponding to one-fourth of the doses of globulin used.

These experiments clearly demonstrate that rabbit's serum, which had no power of producing hemolysis of venomised corpuscles, still contained venom activating constituents which were resident in the globulin fraction. These constituents were, like complement, thermolabile (at 55°C.). In the case of hemolysis with immune body, as already shown, the albumin exerted no inhibitory action (table 2 B).

The globulin fraction of rabbit's serum apparently represents the whole complement of the serum both for immune body and venom but in the case of venom the native serum is inactive in virtue of inhibition by the albumin. It was concluded that the deficiency of the whole serum in this respect was due to the albumin antagonizing or "masking" the activity of the globulin.

TABLE 3

	LYSIS OF 0.5 CC. OF 5 PER CENT OX BLOOD SUSPENSION 0.0005 GRAM COBRA VENOM					
Guinea-pig's complement + Rabbit's "end-piece"........	0.0075 cc. + 0.015 cc. dist.	0.01 cc. + 0.02 cc. mk.	0.02 cc. + 0.04 cc. dist.	0.04 cc. + 0.06 cc. tr.	0.06 cc. + 0.12 cc. f.tr.	0.1 cc. + 0.2 cc. 0
Guinea-pig's complement.....	0.0075 cc. j.c.	0.01 cc. c.	0.02 cc. c.	0.04 cc. c.		
Guinea-pig's complement..... + Guinea-pig's "end-piece".....	0.0075 cc. + 0.015 cc. c.	0.01 cc. + 0.02 cc. c.	0.0 cc. + 0.04 cc. c.	0.04 cc. + 0.08 cc. c.	0.06 cc. + 0.012 cc. c.	0.1 cc. + 0.2 cc. c.

Controls

0.2 cc. guinea-pig's end-piece = 0

0.2 cc. rabbit's end-piece = 0

It was found also that rabbit's serum-albumin inhibited the action of guinea-pig's serum with venom. In the experiment shown (table 3), varying amounts of guinea-pig's complement mixed with quantities of rabbit's albumin (end-piece) representing respectively double these amounts of rabbit serum, were tested in series with 0.5 cc. of ox blood suspension + cobra venom. There was marked inhibition and a zone phenomenon was produced.

Corresponding mixtures of guinea-pig's complement with guinea-pig's end-piece show no inhibition of lysis. This experiment shows an interesting difference in the albumin fraction of these two sera in regard to cobra venom hemolysis.

EXPERIMENTS WITH HUMAN SERUM

Specimens of fresh human sera were also investigated in the light of the findings with rabbit's serum and it was found that the globulin fraction was actively hemolytic in the presence of venom even when the native serum had no action. Sera were fractioned into globulin and albumin by the ammonium sulphate method and the fractions were tested with immune body and with venom; it was observed that the globulin displayed practically the full complementing action of the serum for immune body.

TABLE 4

HUMAN SERUM	LYSIS OF 0.5 CC. OF OX BLOOD SUSPENSION + 0.0005 GRAM COBRA VENOM						
	0.01 cc.	0.025 cc.	0.05 cc.	0.075 cc.	0.1 cc.	0.15 cc.	0.5 cc.
Fresh serum	0	0	0	0	0	0	0
Globulin precipitated by $(NH_4)_2SO_4$ method	0	0	tr.	dist.	mk.	j.c.	
Albumin precipitated by $(NH_4)_2SO_4$ method	0	0	0	0	0	0	
Albumin+Globulin $(NH_4)_2SO_4$ method	0	0	0	0	0	0	

HUMAN SERUM	LYSIS OF 0.5 CC. OF OX BLOOD SUSPENSION + 5 DOSES IMMUNE BODY					
	0.01 cc.	0.025 cc.	0.05 cc.	0.075 cc.	0.1 cc.	0.15 cc.
Fresh serum	mk.	j.c.	c.	c.	c.	c.
Globulin	dist.	v.mk.	c.	c.	c.	c.
Albumin	0	0	0	0	0	0
Albumin+globulin	mk.	al.c.	c.	c.	c.	c.

Controls

0.5 cc. of suspension (no immune body nor venom)+human serum 0.5 cc.=no lysis.

0.5 cc. of suspension (no immune body nor venom)+globulin 0.2 cc.=no lysis.

In the case of venom the action of the globulin was inhibited by the albumin. Table 4 shows the results. In the experiment quoted the mimimum dose of human complement with immune body was relatively small, about 0.025 cc. for 0.5 cc. of the sensitised suspension. Observations were also made as to the power of the albumin of human serum to inhibit the hemolytic action of guinea-pig's serum for venomised corpuscles. For convenience end-piece of human serum separated by carbon-dioxide was employed and it was found to exert a marked inhibiting effect (table 5).

TABLE 5

	LYSIS OF 0.5 CC. OF OX BLOOD SUSPENSION + 0.0005 GRAM COBRA VENOM					
	0.005 cc.	0.01 cc.	0.02 cc.	0.04 cc.	0.06 cc.	0.1 cc.
Guinea-pig's serum........	j.c.	c.	c.	c.	c.	c.
Human "end-piece"......	0	0	0	0	0	0
Guinea-pig's serum........	0.005 cc.	0.01 cc.	0.02 cc.	0.04 cc.	0.06 cc.	0.1 cc.
+	+	+	+	+	+	+
Human "end-piece"......	0.005 cc.	0.01 cc.	0.02 cc.	0.04 cc.	0.06 cc.	0.1 cc.
	mk.	mk.	dist.	dist.	dist.	tr.
Guinea-pig's serum........	0.005 cc.	0.01 cc.	0.02 cc.	0.04 cc.	0.06 cc.	0.1 cc.
+	+	+	+	+	+	+
Human "end-piece"......	0.01 cc.	0.02 cc.	0.04 cc.	0.08 cc.	0.12 cc.	0.2 cc.
	mk.	tr.	0	0	0	0

EXPERIMENTS WITH HORSE'S SERUM

Certain experiments with horse serum yielded very interesting results. As regards the hemolysis of ox corpuscles in the presence of venom, horse serum often exhibits a powerful activating effect and this property is partially retained even when the serum is heated to a temperature of 56°C.

It is apparent therefore that this activating power is not entirely due to serum constituents of complement nature.

Kyes (10) assumed that venom activation by serum was entirely due to the lecithin present in the serum and that the lipoid existed in combination with the serum proteins. In the case of horse serum he suggested that the lecithin was very lightly bound and was therefore available in the fresh serum.

Horse serum has no complementing power for ox's corpuscles along with immune body (see Muir (11)).

The globulin and albumin were separated by the ammonium sulphate method and tested with venom and immune body.

TABLE 6

HORSE SERUM	LYSIS OF 0.5 CC. OF 5 PER CENT OX BLOOD SUSPENSION													
	+ 0.0005 gram cobra venom							+ 5 doses of immune body						
	0.01 cc.	0.025 cc.	0.15 cc.	0.1 cc.	0.02 cc.	0.3 cc.	0.5 cc.	0.01 cc.	0.025 cc.	0.05 cc.	0.1 cc.	0.2 cc.	0.3 cc.	0.5 cc.
Fresh serum	j.c.	c.	c.	c.	c.	c.	c.	0	0	0	0	0	0	0
Globulin $(NH_4)_2SO_4$ method	tr.	j.c.	c.	c.	c.	c.	c.	0	0	0	0	0	0	0
Albumin $(NH_4)_2SO_4$ method	dist.	c.	c.	c.	c.	c.	c.	0	0	0	0	0	0	0
Globulin+albumin $(NH_4)_2SO_4$ method	j.c.	c.	c.	c.	c.	c.	c.	0	0	0	0	0	0	0

HORSE SERUM	+ 0.0005 gram cobra venum (70°C.)					
	0.01 cc.	0.025 cc.	0.05 cc.	0.1 cc.	0.2 cc.	0.3 cc.
Globulin $(NH_4)_2SO_4$ method	0	0	0	0	ftr	
Albumin $(NH_4)_2SO_4$ method	dist.	c.	c.	c.	c.	
Globulin+albumin $(NH_4)_2SO_4$ method	dist.	c.	c.	c.	c.	

Controls: No immune body nor venom.

Globulin $\left\{\begin{array}{l}0.25 \text{ cc.} = \text{no lysis.}\\ 0.5 \text{ cc.} = \text{no lysis.}\end{array}\right.$

Fresh serum: 0.5 cc. = no lysis.

It was found that both the globulin and albumin individually displayed marked activating powers and the combination of the two fractions represented the full hemolytic power of the serum, of course only by a process of summation of effects (table 6).

To ascertain whether the serum lecithin played some part in producing lysis along with venom, these two fractions both together and separately were tested with ox corpuscles + cobra venom which had been heated to 70°C. for one-half hour according to the method of Morgenroth and Kaya (12), who showed that with heated venom, lecithin is actively hemolytic while complement is inactive.

It was found on carrying out these experiments that the globulin fraction was quite inert with heated venom while the albumin was as active as with fresh venom (table 6); the lecithin nature of the activating constituents of the albumin fraction was thus demonstrated.

While therefore lecithin bodies play some part in the activating effect of horse's serum, other constituents probably of complement nature are equally concerned. It is of interest also to note that the lecithin substance should be associated with the albumin fraction. The other activating elements are contained in the globulin.

DISCUSSION AND CONCLUSIONS

These experiments elicit striking differences in the constitution of the complements of different animals apart from their relative activity with hemolytic immune body and venom.

In the case of human and rabbit's serum acting on ox red blood corpuscles + immune body or venom, the complement is entirely associated with the globulins of the serum while in the case of guinea-pig's serum which represents with these hemolytic systems a much more powerful complement, the albumin fraction is also an essential constituent of the complement.

Whether the potency of a complement depends on the presence of constituents associated with the serum albumin is a matter for further investigation.

In the case of human and rabbit's sera, however, acting with venom, the effect of the globulin is "masked" in the whole serum by the albumin while in the case of guinea-pig's serum the albumin also contributes to the full action of the serum along with the globulin.

It has also been shown how the albumin of human and rabbit's serum may inhibit the action of guinea-pig's serum globulin.

In the case of horse's serum the activating effect with venom is due not only to a complement body represented by the globulin but also to the lecithin contained in the albumin fraction.

REFERENCES

(1) Stephens, J. W. W.: Jour. Path. and Bacter., 1900, **6**, 273.
(2) Browning, C. H., and Mackie, T. J.: Jour. Path. and Bacter., 1912, **17**, 120; Biochem. Zeitsch., 1912, **43**, 229; Zeitsch. f. Immunitäts., Orig., 1913, **17**, 1.
(3) Ferrata, A.: Berl. klin. Woch., 1907, **44**, 366.
(4) Brand, E.: Berl. klin. Woch., 1907, **44**, 1075.
(5) Liefmann, H.: Münch. med. Woch., 1909, **56**, 2097.
(6) Sachs, H.: Handbuch d. Tech. & Meth. d. Immunitäts., 1909, **2**, 969.
(7) Omorokow, L.: Zeitsch. f. Immunitäts., Orig., 1911, **10**, 285.
(8) Browning, C. H., and Mackie, T. J.: Zeitsch. f. Immunitäts., Orig., 1914, **21**, 422.
(9) Chick, H., and Martin, C. J.: Biochem. Jour., 1913, **7**, 380.
(10) Kyes, P.: Jour. of Infect. Dis., 1910, **7**, 181.
Ehrlich, P.: Studies on Immunity, 2d ed., 1910, p. 291.
(11) Muir, R.: Jour. Path. & Bacter., 1912, **16**, 523.
(12) Morgenroth, J., and Kaya, R.: Biochem. Zeitsch., 1908, **8**, 378; Biochem. Zeitsch., 1910, **25**, 88.

ON THE PLACENTAL TRANSMISSION OF SO-CALLED NORMAL ANTIBODIES

II. ANTITRYPTIC-ACTING BODIES

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In 1902 Halban and Landsteiner (1) observed that the blood of child-bearing mothers contained a greater amount of antitryptic-acting bodies than the funicular blood of their offspring, and in 1909 this question was taken up nearly simultaneously by Graefenberg (5), Becker (6), v. Reuz (7) and also Lust (8). They all determined the antitrypsin content of the blood by means of v. Bergmann and Bamberg's (3) and Kurt Meyer's (4) modification of the Gross-Fuld (2) casein method. Graefenberg found that the antitrypsin titer was nearly doubled during pregnancy, and then again became normal shortly after parturition. Becker examined twenty-five new-born children and their mothers with the result that titers of the new-born were on an average nearly the same as that ordinarily encountered in adults; on the other hand they were somewhat lower than those of the mothers, the titer of the latter being considerably heightened. v. Reuz examined the blood of diseased as well as healthy children, from six days to eleven months old, and reported that the values were all very low before the onset of the sickness. Lust examined the blood of ten normal children, from fourteen days to more than two years old, and found the titers almost as high as in adults.

Gammeltoft (9) improved the casein-method by undertaking his measurements with a casein solution of a special hydrogen-ion concentration, and by determining the nitrogen which might be titrated with formol during the process. He corroborated the presence of the greater antitryptic power of the mother's serum as compared with the corresponding funicular serum of the children and also the increase during pregnancy, and he further found that during the same period no increase took place in gravid cows and rabbits. Gammeltoft supposes that the antitryptic action is due to lipoid compounds, and bases this supposition upon the fact that these bodies, as demonstrated by Bauer and others, can be shaken off with ether, and also that the bodies in question are suspended. Gammeltoft gives in conclusion the observations made by Morgagni, Hunter and Virchow, that the serum of gravid females is more opalescing than is serum ordinarily, and that this opalescence, according to Virchow, is due to phosphorated fat. In my own experiments, also, there was an apparent correlation of opalescence and enhanced antitryptic power of a serum; for the serum of kids was generally opalescing, so much so, in fact that on standing a cream-like layer might form on the surface, whereas the serum of the mother animal, before as well as after parturition, was usually clear; and, in concurrence with this difference in appearance, the serum of kids, as will be shown later, was found to inhibit tryptic action more strongly than did the maternal serum.

The antitryptic action of the serum was tested by the writer in seven goats and their kids, among which were three pairs of twins; of the latter one kid, though normal, had to be killed immediately after birth, and as the first sample from the corresponding twin was lost, the sample of the killed kid made a good supplement. The first blood sample was taken from the jugular vein, immediately after birth and before the kids had taken nourishment. All test samples were subsequently taken from the jugular vein and at the same hours.

The measurements of the antitryptic power were determined by means of the Gross-Fuld method, as modified at the Serum-Institute for quantitative use. First the entirely digestive dose of trypsin was tested against 2 cc. casein solution (1 gram casein dissolved in 100 cc. $\frac{N}{10}$ NaOH neutralized with $\frac{N}{10}$ HCl and diluted with a 0.9 per cent dilution of sodium chloride to 500 cc.). Based upon this preliminary experiment a suitable excess of trypsin was used in the actual experiment, which was made in the following manner: to a series of test tubes decreasing doses of serum were added (with intervals of 20 per cent), after the suitable quantities had first been determined by means of preliminary experiments. Following upon the volumetric correction of the contents of the tubes (total volume 1 cc.) the trypsin-solution was added, after which the tubes were left for twenty minutes in a waterbath (37°C.). After they had been cooled with running water 2 cc. casein solution per tube was added, and, after being well shaken, they were again placed at 37°C. for three quarters of an hour. The tubes were then cooled once more and 0.1 cc. acetic acid solution (5 cc. acetic acid + 45 cc. absolute alcohol + 50 cc. water) was added. Thereafter a tube with a definite opalescence was chosen and in each of the series the tube with corresponding opalescence was found. The measure was repeated with another tube with a different degree of opalescence. The mean of the reciprocal values of these two measurements are the figures given in the tables.

A comparison between the figures of the table is only permissible within the same experiment; i.e., a mother animal with corresponding kids, seeing that all the samples originating from a group of this kind were measured at the same time. Furthermore, the series with decreasing doses from each serum sample, including the eventual minima and maxima of the antitryptic action, extended over such a range that they embraced the same serum dosage for both mother and kid, and thus the effect of equal doses of serum against the same quantity of casein solution and in conjunction with the application of like dosage of the same trypsin solution was observed.

The method of Gammeltoft (9) is more expedient, in so far as various experiments might be compared from a quantitative point of view, but it is difficult to use it when, as in this case, we are dealing with long series of many serum samples, and the Gross-Fuld method seemed to me to offer a sufficient foundation for my purpose, which was in isolated experiments to determine the relative proportion of antitryptic-acting bodies in the blood of the mother animal and the kids, and their fluctuations during the experimental period. Finally it turned out that repetitions gave satisfactory results.

As regards the titers found, it appears from the table that the titers of the kids, as contrasted with the results of the other ex-

TABLE 1

Titration of antitrypsin in maternal and kids' serums

Dates of taking serum samples. *Birthdays. a, Titer found directly; b, weight factor; c, titer correlated to weight.

MOTHER OF KID	MOTHERS' SERUM												
1♂	2/1 100	*5/2 140	6/2 140	7/2 140	9/2 160	11/2 200	14/2 170	16/2 170	18/2 160	21/2 156			
2♂	20/12 77	*5/2 130	6/2 105	7/2 138	9/2 138	11/2 138	14/2 120	16/2 138	18/2 138	21/2 125			
3♀	20/12 100	6/2 175	*9/2 175	10/2 175	11/2 167	12/2 175	14/2 175	16/2 175	19/2 182	21/2 154	25/2 160		
4♀	12/2 80	*14/2 80	16/2 83	21/2 83	25/2 83	28/2 71	4/3 70	11/3 70	17/3 68				
6♂	12/2 80	*21/2 95	23/2 105	25/2 128	28/2 138	3/3 125	7/3 110	11/3 87	16/3 90				
7♂ 8♂	6/2 98	12/2 115	*21/2 91	23/2 132	25/2 141	28/2 91	3/3 89	7/3 96	11/3 117	16/3 121	20/3 87	27/3 94	
9♂ 10♀	6/2 59	12/2 79	*24/2 78	26/2 105	28/2 82	3/3 87	7/3 85	11/3 85	14/3 77	17/3 77	21/3 68	25/3 67	30/3 63

MOTHER OF KID			KIDS' SERUM										
1♂			*5/2	7/2	9/2	11/2	18/2	21/2	25/2				
		a	200	222	166	174	166	182	176				
		b				1.133	1.44	1.60	1.773				
		c				197	239	291	312				
2♂			*5/2	7/2	16/2	21/2	25/2						
		a	245	182	156	217	208						
		b			1.307	1.40	1.493						
		c			204	304	300						
3♀			*9/2	10/2	11/2	12/2	14/2	17/2	21/2	25/2			
		a	286	244	286	286	222	216	182	175			
		b			1.0	1.0	1.061	1.182	1.303	1.439			
		c			286	286	236	255	237	252			
4♀			*14/2	16/2	18/2	21/2	25/2	28/2	4/3	9/3	11/3	17/3	
		a	133	126	124	125	111	87	100	90	110	110	
		b		1.119		1.343	1.60	1.789	1.983	2.269		2.737	
		c		141		168	178	156	198	204		301	
6♂			*21/2	23/2	25/2	28/2	3/3	7/3	11/3	16/3			Kid 5
		a		100	125	95	88	78	76	67			*21/2
		b			1.204	1.385	1.555	1.838	2.053	2.389			a174
		c			151	132	137	143	156	160			
7♂ 8♂			*21/2	23/2	25/2	28/2	3/3	7/3	11/3	16/3	21/3	27/3	
	7	a	187	168	164	131	107	107	88	82	83	72	
		b		1.123	1.26	1.411	1.561	1.712	1.821	2.011	2.228	2.519	
		c		189	207	185	167	183	160	165	185	181	
	8	a	202	157	126	113	121			134	131	131	
		b		1.295	1.422	1.567	1.756			2.302	2.564	2.902	
		c		203	179	177	213			309	336	380	
9♂ 10♀			*24/2	26/2	28/2	3/3	7/3	11/3	14/3	17/3	21/3	25/3	30/3
	9	a	250	166	123	108		88	83	73	77	72	96
		b		1.161	1.40	1.606		2.206	2.333	2.556	2.861	3.167	3.528
		c		193	172	173		194	194	187	220	228	339
	10	a	222	148	141	108	88	80	77	80	68	72	67
		b		1.108	1.332	1.462	1.614	1.848	1.951	2.085	2.309	2.525	2.803
		c		164	188	158	142	148	150	167	157	182	188

Note. As regards kids 1 and 2 I had to content myself by taking as my starting point the weight on the fourth day after birth. These kids were very large and grew with unusual rapidity, which is not expressed in the weight-factor.

perimentators on human beings and other animals than those used for my experiments, in all cases are higher than those of the mother animals; whereas the increase in the titer of the mother animal, which has likewise been demonstrated in all of the cases, agrees with the findings of other investigators. In some of the mother-animals this increase continues after birth, following which—as in human beings—a decrease takes place; whereas in others the titer remains constant through the whole of the experimental period (up to somewhat more than a month).

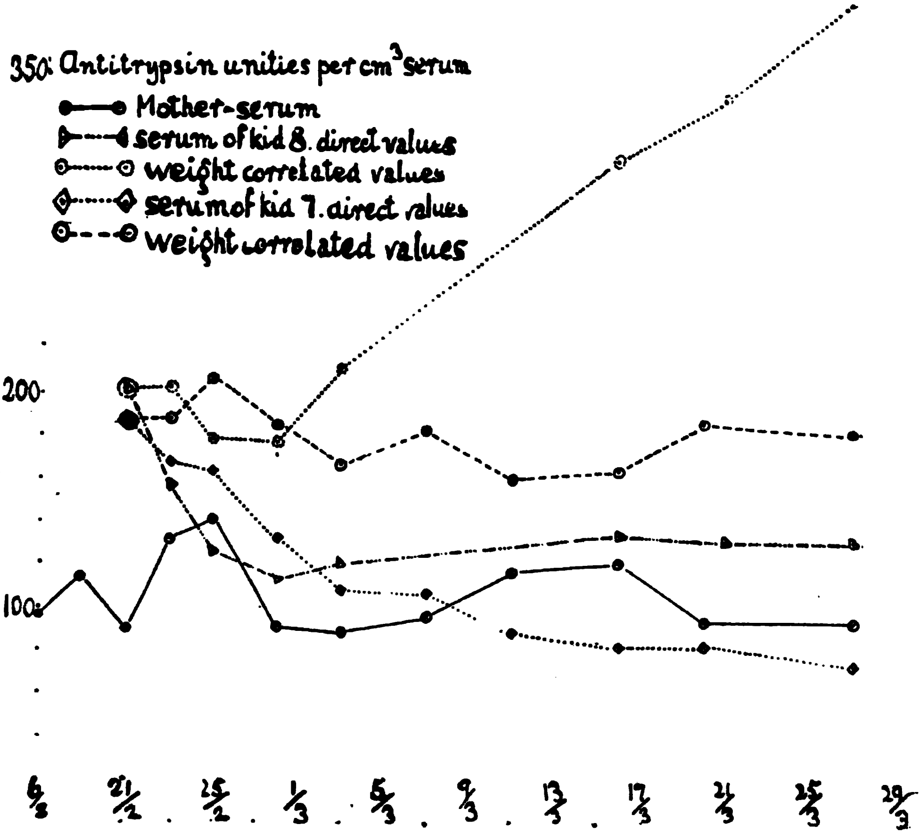

CHART 1

From the values which in the table are marked "a;" namely the directly found titers, it appears that in the kids there is in most cases a gradual decrease of the antryptic acting bodies after birth; if, however, one looks at the weight-correlated values marked "c" (found by dividing the weight at birth into the weight of the moment and multiplying the titers with the resulting factor marked "b") it will be noted that, after some fluctuation, there is an increase, a constancy or in a single case a decrease in the titers. This decrease occurred in a kid (no. 3) which from the first throve badly. It will be noted that the increase observed in the case of the weight-correlated titers may be somewhat different, even in the case of twins (7 and 8, 9 and 10), for it appears from the weight factors given that of 7 and 8 (which by the way had very nearly the same weight at birth, that is, 2850 and 2750 grams respectively) 8 throve least in the beginning and its titer at the same time sank below that of 7; later on the case was reversed, both as regards titer and growth. Of kids 9 and 10, the weights of which at birth were 1800 and 2230 respectively, 9 grew proportionally more rapidly than 10, and its weight-correlated curve also rose more quickly.

If one compares the weight-correlated figures, it appears that five of the examined kids have an increasing and only one a decreasing titer, whereas in three it is nearly constant, so that the total quantity of the antitryptic acting bodies in the cases examined on an average has increased.

The fact that the increase and decrease of the titer in certain cases have turned out to follow growth or failure to grow might, as suggested by other experimentators, indicate that we are here dealing with a fat- or lipoid-action.

SUMMARY

By examinations of the proportion between the amount of antitryptic-acting bodies in the blood of goats and their newborn kids, the titers of the kids were in all cases found to be higher than those of the mother animals.

The titers of the mother animal as a rule increase before parturition.

There seems to be some connection between the growth of the kids and the antitryptic-acting power of the blood, so that the titer of the kid decreases when it thrives badly, and increases when it thrives well. which would agree with the supposition set forth by previous experimentators, that the antitryptic action is connected with a fat or lipoid effect.

REFERENCES

(1) HALBAN AND LANDSTEINER: Münch. med. Wochenschr., 1902, **49**, 473–476.
(2) GROSS: Archiv f. exper. Pathol., 1907, **58**, 157–166.
(3) v. BERGMANN AND BAMBERG: Berliner klin. Wochenschr., 1908, **45**, 1396.
(4) v. BERGMANN AND MEYER, KURT: Berliner klin. Wochenschr., 1908, **45**, 1673–1677.
(5) GRAEFENBERG: Münch. med. Wochenschr., 1909, **56**, 702–704.
(6) BECKER: Berliner klin. Wochenschr., 1909, **46**, 1016–1017.
(7) v. REUSS: Wiener klin. Wochenschr., 1909, **22**, 1171–1172.
(8) LUST, M.: Münch. med. Wochenschr., 1909, **56**, 2047–2051.
(9) GAMMELTOFT, S.: Meddelelser fra Rigshospitalet, Copenhagen, 1912.

SIMPLIFICATION AND PARTIAL REVISION OF THE FACTORS INVOLVED IN THE COMPLEMENT FIXATION TEST FOR INFECTIOUS ABORTION IN CATTLE

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The applicability of the complement fixation test in the diagnosis of infectious abortion was demonstrated by Holth (1909) whose observations were soon substantiated by Wall (1911), Mohler and Traum (1911), Surface (1912), Hadley and Beach (1912) and others. Holth made a comparative study of complement fixation and agglutination and found that both of these tests were specific for infection by the Bang bacillus. They have since then been regarded by most investigators as reliable and of much value in the hands of competent technicians. Considerable criticism has been aimed, however, in recent years at the complement fixation test because of the alleged difficulties that were encountered in the carrying out of the test, which has led in a few known instances at least to its abandonment and sole reliance on the agglutination reaction.

Both the agglutination and the fixation reactions are invaluable methods in the scientific study of the complicated problem of infectious abortion. They have been employed in the present joint investigation of the Sheffield Scientific School Bacteriological Laboratory and the Storrs Agricultural Experiment Station for almost six years, during which period over 4500 blood samples have been tested. With very few exceptions, the two tests have served excellently as checks for each other, and it is for this reason alone that they have been and are still regarded as equally indispensable.

It cannot be denied that the carrying out of the complement fixation test as it has been developed in the past is beset with difficulties which only the most careful and competent operator is able to surmount. It has been the purpose of this study, therefore, to attempt clarification of the phases which apparently have given the largest amount of trouble.

The fixation test for infectious abortion in cattle has undergone but little, if any, real modification since the time of Holth and Wall. There has been some variation in the methods of preparing the antigen; some laboratories have adhered to the serum broth culture method, while others have substituted agar medium, with or without the fresh serum, for the bouillon. The greatest difficulty seems to have been encountered in the preparation and titration of the bacterial antigen.

In the regular fixation test the complement is obtained from the guinea-pig, and the hemolytic amboceptor from rabbits that have been immunized against washed sheep's corpuscles, which are employed also as the hemolytic antigen in the test. Four different amounts of cow's serum are as a rule used, namely 0.2, 0.1, 0.05 and 0.02 cc. The cow's serum, complement and bacterial antigen are mixed with 1.5 cc. of physiological saline solution and incubated for one hour preliminary to the addition of the hemolysin and sheep's corpuscles. The final incubation is for two hours. For a complete description of the technic of the complement fixation test in infectious abortion, including the preliminary standardization of complement, hemolysin and antigen, the reader is referred to the work of Surface (1912), Hadley and Beach (1912) and Rettger and White (1918).

The only modifications of any importance which have been made in connection with the present investigation of infectious abortion were the adoption of the Wenner (1918) method of bleeding guinea pigs, the preservation of sheep's corpuscles by the Bernstein and Kaliski method of formalinizing (1912), and the intravenous injection of small but increasing doses of washed, undiluted sheep's corpuscles in rabbits for the purpose of more rapid and certain hemolysin production.

The chief aim in the present investigation has been to simplify and standardize the technic of the complement fixation test for infectious abortion in cattle. The main emphasis has been placed on the preparation and titration of the antigen, though the other factors involved in the fixation scheme have received their due share of attention.

COMPLEMENT

During the past two years the Wenner (1918) method of bleeding guinea-pigs has been employed in this laboratory. During this period the loss of animals from injury sustained in the operation has been negligible. In many instances blood was drawn from the same guinea pigs repeatedly without any apparent untoward effect on them, and without any decreased complement potency of the serum. The saving of guinea-pigs by this method was a source of much satisfaction. It may be said without exaggeration that a very small number of large guinea pigs which are used solely for furnishing complement will supply enough serum for routine daily fixation tests, and that if facilities for breeding are reasonably good there will be little, if indeed any, occasion for replenishing the stock.

The description of the method of drawing blood, as given by Wenner, was as a matter of necessity somewhat incomplete. For this reason it has been regarded by some technicians as perhaps crude and impracticable. An effort has been made in the present work to refine the technic and to make it so simple that even the unskilled operator can employ it successfully. As now conducted in this laboratory the technic should be acquired easily. The following is a complete description of the method, including the present refinement.

A large guinea-pig is selected for the operation, preferably a young healthy male. The animal is suspended from a support by means of a cord drawn around the hind legs. The head is immediately grasped with the left hand so that the thumb comes under the lower jaw, and the body is turned ventral side up. In this way the animal is easily controlled by very light pressure. The head is turned back at right angles to the dorsal aspect, the hair on the neck clipped as close to the skin as possible, and the bared neck washed with 2 per cent solution of cresol followed by 50 per cent alcohol. It is well to rub the neck briskly with absorbent cotton until it is practically dry.

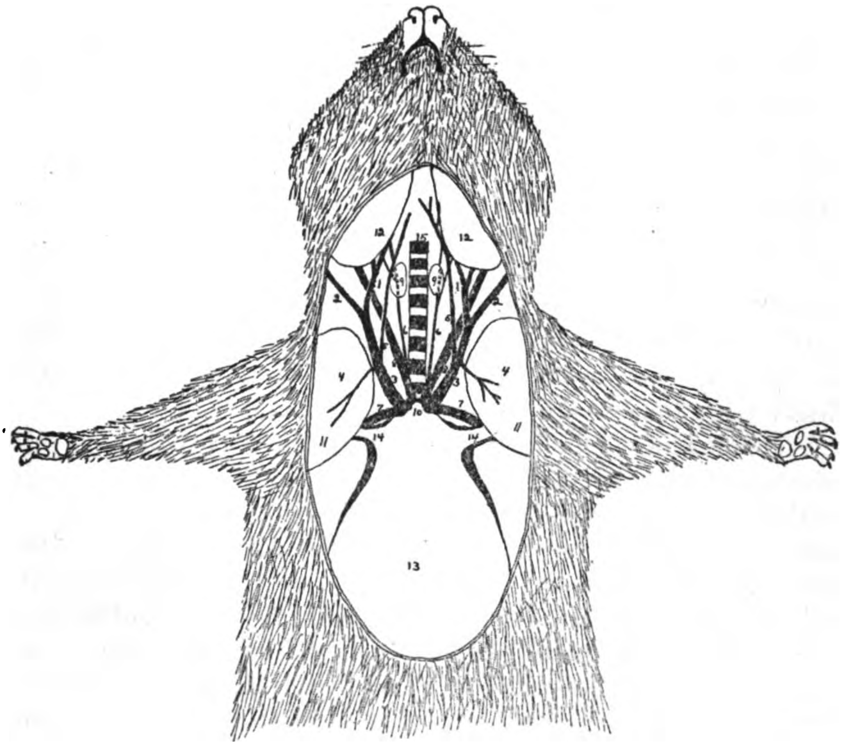
The guinea-pig should be etherized, of course, but only sufficient anaesthesia should be given to make the animal insensible to pain and the period during which it is under the influence of the ether must be made as short as possible.

As soon as the vein from which the blood is to be secured is located a transverse incision 10 to 15 mm. long is made with a pair of sharp surgical scissors in the skin over the sternomastoid muscle about half way between the masseter and subscapularis muscles. The vein is then quickly brought to view and partly or completely severed with the same instrument. The guinea pig is lowered instantly to allow the blood to drain from the wound into a sterile Petri dish or centrifuge tube, without touching any part of the body.

The vein which is chosen for the operation (see diagram) is an anterior ventral branch of the external jugular vein, and corresponds in location to the anterior jugular vein of the higher vertebrates. It lies above the sternomastoid and the sternohyoid muscles, but under the superficial muscles of the neck. When it is cut while the animal is suspended at full length the blood backs into it from the large external jugular vein and any desired amount can be collected, in fact more blood can usually be obtained in this way than by severing the external jugular vein and bleeding the guinea-pig to death. The blood runs slowly but steadily as long as the position of the animal is maintained.

As soon as the required amount of blood is obtained the guinea pig is placed on its back on the table, and several stitches are sewed through the loose edges of the cut skin. The flow of blood quickly stops. After washing the wound with dilute cresol or with alcohol the animal is returned to its cage. In the course of two or three days the wound is completely healed and the pig appears as strong as ever.

The preparation of the serum from the blood involves the usual technic. The blood is allowed to clot thoroughly, preferably in the refrigerator. The clot is broken up and the Petri dish returned to the cooler for at least four or five hours. A clear or slightly opalescent straw-colored serum is usually obtained which, with rare exceptions, has good complementary properties. When prepared for immediate use no attempts are made to preserve it except by refrigeration in the ordinary ice box.



1. Anterior facial veins
2. Posterior auricular veins
3. External jugular veins
4. Cephalic veins
5. Anterior jugular veins
6. Internal jugular veins
7. Subclavian veins
8. Sternomastoid muscle
9. Thyroid glands
10. Manubrium
11. Subscapularis muscles
12. Masseter muscles
13. Pectoral muscles
14. First pectoral muscle
15. Trachea

Both the Rhamy (1918) and the Noguchi (1918) methods of preserving the complement have been employed by the writer, with considerable success, but mostly the former. The Rhamy method, as here modified, consists in adding 40 per cent of a 12 per cent solution of sterile sodium acetate to the serum. The treated serum is allowed to remain in the ice box for two or three days before using. At the end of this period the titer of the complement is rather high, but the complement does not very materially weaken for a period of three or four weeks.

HEMOLYSIN

For the production of hemolysin Coca (1915) advocates intravenous injection of three rabbits with 1 cc. of the washed sheep's corpuscles. Two injections are made, the intervening period being five days. The animals are bled five days after the second injection and the hemolytic properties determined. Coca figures that at least one of the three rabbits will furnish potent hemolysin. Stitt (1918) injects intravenously 1 cc. of a 10 per cent erythrocyte suspension, and five days later 2.5 cc. of the strength suspension. A third and last injection, this time 5 cc., is given five days after the second. Seven or eight days later 4 or 5 cc. of blood are drawn from the ear and the titer determined.

The following method has been used for over two years in this laboratory with excellent results. One-half to 1 cc. of well-washed sheep's corpuscles is injected into the marginal vein of each of two rabbits; one or two days later 1 cc. of the same material is applied in the same manner, and on the third or fourth day after the first treatment a final dose of 1.5 to 2.0 cc. of washed red cells is administered. Blood is drawn from the marginal ear vein four or five days after the last injection, for the potency test. If the titer is satisfactory one or both rabbits are killed not later than the tenth day after the third treatment. If a serum of still greater potency is desired a fourth injection (1.5 to 2 cc. of the corpuscles) is given, and four or five days later the titer again determined. Formalinized corpuscles can be used for the production of the hemolytic amboceptor providing they are not more than a week old and are thoroughly washed with sterile physiological saline solution just before using (see page 405).

Occasionally a rabbit is lost through anaphylaxis. For this reason, partly, two rabbits are chosen for the hemolysin production. The use of more than one rabbit also increases the certainty of obtaining a hemolytic serum of the desired strength, though we have never failed to produce a satisfactory serum by the method just described. With rare exception, the serum has been so active that a dilution of 1 to 100 has been necessary in order to determine the titer with any degree of accuracy. Even with this dilution the exact titer has usually fallen between 0.01 and 0.05.

SHEEP'S CORPUSCLES

The method of preserving sheep's erythrocytes described by Bernstein and Kaliski (1912) and later by Wenner (1918) has been employed with considerable success for the past two years in this laboratory.

Immediately after the blood has been drawn from the jugular vein of the sheep and defibrinated in a sterile bottle containing shot or glass beads it is strained through absorbent cotton and thoroughly mixed with a 40 per cent (commercial) solution of formaldehyde in the proportion of 1 cc. of the undiluted formaldehyde to 800 cc. of the defibrinated sheep's blood. When preserved in this manner the erythrocytes will keep, if held at a uniform temperature in a refrigerator, for fully three weeks at least. It is very important that the bottle, filtering funnel and everything else with which the blood comes in contact be sterile.

The corpuscles are washed when needed. The desired amount of blood is then withdrawn from the bottle with a sterile pipette and the corpuscles precipitated in the centrifuge and washed with physiological saline solution in the usual way. For titration work and for the final complement fixation tests 2 per cent suspensions of the washed corpuscles are made in saline solution.

BACTERIAL ANTIGENS

The preparation and titration of bacterial antigens have presented greater difficulties than any other phase of the complement fixation test for infectious abortion. It is largely because of these difficulties that the test has been abandoned in many laboratories. Very little has been done in past years to simplify this part of the general technic.

The best success in the preparation of *B. abortus* antigen has been attained in this laboratory by the use of slant agar cultures. It has been quite apparent that the choice of peptone for the agar medium upon which the organism is grown is an important factor; also that temperature and period of incubation must be taken into account seriously.

In the present investigation an attempt was made to devise a uniform and reliable method of antigen production. This involved a study of the influence of age of bacterial cultures, different brands of peptone, different strains of *B. abortus*, and of the initial hydrogen ion concentration of the medium, on the antigenic properties of the bacterial growths.

1. Relation of age of B. abortus culture to antigenic potency

In the following experiments the antigen was prepared from slant agar growths of *B. abortus*, Bang strain, which were incubated at 37°C. under aerobic conditions. Fairchild's peptone (1.0 per cent) was employed. The growths were washed from the agar with physiological saline solution, and the bacterial suspensions filtered through absorbent cotton. The suspensions were shaken vigorously in glass-stoppered bottles for thirty minutes, after which they were heated in a water bath at 62°C. for one hour, with the bottles submerged to the neck. Immediately following thorough cooling the suspensions were carbolyzed (0.5 per cent). From these stock suspensions the required dilutions were made and the antigen titers determined. The agar used in the preparation of the different antigens was as nearly uniform in composition and reaction as possible.

In the first experiment the antigen (a) was prepared from agar cultures which had been incubated at 37°C. for four days and then kept in a dark cool closet (15 to 18°F.) for six weeks. The final suspension was diluted 1:5 with carbolized saline solution. Table 1 is a record of the results.

TABLE 1

	ANTIGEN (a)													
	With immune abortion serum						Without immune serum							
	0.01 cc.	0.03 cc.	0.05 cc.	0.08 cc.	0.1 cc.	0.2 cc.	0.05 cc.	0.1 cc.	0.2 cc.	0.3 cc.	0.4 cc.	0.5 cc.	0.6 cc.	0.8 cc.
Hemolysis.....	+	?	–	–	–	–	+	A	A	A	A	A	A	+

+ indicates complete hemolysis, – absence of hemolysis, and A anticomplementary properties.

The titer of the antigen is 0.05, and from this standpoint quite satisfactory, but anticomplementary factors are encountered in as low a dilution as 0.1, which in itself renders this antigen useless for the complement fixation test which, according to the technic very generally employed, calls for 4 units of antigen, or in this case 0.2 cc.

In the next experiment antigen (b) was prepared from cultures which had been incubated for five days at 37°C. and then kept in the cool dark closet for 10 days. The stock suspension was diluted 1:10 and titrated, with the results shown in table 2.

TABLE 2

	ANTIGEN (b)													
	With immune abortion serum						Without immune serum							
	0.01 cc.	0.03 cc.	0.05 cc.	0.08 cc.	0.1 cc.	0.2 cc.	0.05 cc.	0.1 cc.	0.2 cc.	0.3 cc.	0.4 cc.	0.5 cc.	0.6 cc.	0.8 cc.
Hemolysis.....	+	+	–	–	–	–	+	+	A	A	A	–	–	–

The titer of this antigen (b) is 0.05, which means that 0.2 cc. of the antigen would be needed for the regular fixation test, at the concentration of which anticomplementary action of this suspension begins to assert itself. This antigen is, therefore, highly unsatisfactory.

The next antigen (c) was derived from agar cultures that were incubated for six days at 37°C. At the end of this period the growths were promptly removed from the medium and converted into the final antigen preparation. The stock suspensions were diluted 1:5.

TABLE 3

	ANTIGEN (c)													
	With immune abortion serum						Without immune serum							
	0.01 cc.	0.03 cc.	0.05 cc.	0.08 cc.	0.1 cc.	0.2 cc.	0.05 cc.	0.1 cc.	0.2 cc.	0.3 cc.	0.4 cc.	0.5 cc.	0.6 cc.	0.8 cc.
Hemolysis.....	+	+	–	–	–	–	+	+	A	A	–	–	–	–

The titer is again 0.05, and anticomplementary factors were encountered in 0.2 cc. of the suspension. This antigen, also, is useless on account of its marked anticomplementary properties.

The last antigen of this series (d) was prepared from agar cultures (Fairchild peptone) which had been incubated at 37°C. for only four days and then immediately washed off with the saline solution and converted into the stock antigen. The final dilution was 1:5.

TABLE 4

	ANTIGEN (d)													
	With immune abortion serum.						Without immune serum.							
	0.01 cc.	0.03 cc.	0.05 cc.	0.08 cc.	0.1 cc.	0.2 cc.	0.05 cc.	0.1 cc.	0.2 cc.	0.3 cc.	0.4 cc.	0.5 cc.	0.6 cc.	0.8 cc.
Hemolysis.....	+	+	–	–	–	–	+	+	+	+	+	+	+	A

The titer in this instance is 0.05, while the anticomplementary factors are practically nil. Only a slight inhibition of hemolysis was observed in the last tube, that is the 0.8 cc. dilution. This antigen was employed in several complement fixation tests with very satisfactory results. Subsequent work on antigen production has fully corroborated these findings.

The results of experiments thus far indicate that the time factor is an important one in the preparation of *B. abortus* antigen for the complement fixation test in infectious abortion. The cultures should not be incubated longer than four to five days, and the growths should be converted at once into the final carbolized suspension, which must of course be kept at ice box temperature to preserve its antigenic potency.

2. Comparative study of antigens obtained by the use of different brands of peptone

These experiments involved the use of three well-known American brands of peptone, Fairchild's and two others which will be designated here as A and B. Witte's peptone was not included because repeated attempts to develop good antigen on agar containing this peptone had given very unsatisfactory results.

The preparation of the different antigens was carried out in the same way as has already been described (pages 405–406). Incubation was for four days, at 37°C. In addition, the turbidity of the bacterial suspensions was controlled by the use of the McFarland nephelometer (1907), and an effort was made to have an agar medium with a hydrogen ion concentration as near P_H 6.8–6.9 as possible. Tables 5, 6 and 7 are self explanatory.

TABLE 5

Giving the results (titration figures) for antigen grown on agar containing peptone A

	ANTIGEN													
	With immune abortion serum						Without immune serum							
	0.01 cc.	0.03 cc.	0.05 cc.	0.08 cc.	0.1 cc.	0.2 cc.	0.05 cc.	0.1 cc.	0.2 cc.	0.3 cc.	0.4 cc.	0.5 cc.	0.6 cc.	0.8 cc.
Hemolysis.....	+	+	+	−	−	−	+	+	+	+	+	A	A	A

The antigenic titer is 0.08, and anticomplementary interference begins at 0.5 cc. The growths on the agar medium were only moderate.

TABLE 6

Giving the titration figures for antigen prepared by the use of peptone B

	ANTIGEN													
	With immune abortion serum						Without immune serum							
	0.01 cc.	0.03 cc.	0.05 cc.	0.08 cc.	0.1 cc.	0.2 cc.	0.05 cc.	0.1 cc.	0.2 cc.	0.3 cc.	0.4 cc.	0.5 cc.	0.6 cc.	0.8 cc.
Hemolysis.....	+	+	−	−	−	−	+	+	+	+	+	A	A	A

The antigenic titer is 0.05, with anticomplementary action setting in at 0.5. The growths on agar were scant, and the amount of suspension was small, therefore.

TABLE 7

Giving titration figures for antigen grown on agar containing Fairchild's peptone

	ANTIGEN													
	With immune abortion serum						Without immune serum							
	0.01 cc.	0.03 cc.	0.05 cc.	0.08 cc.	0.1 cc.	0.2 cc.	0.05 cc.	0.1 cc.	0.2 cc.	0.3 cc.	0.4 cc.	0.5 cc.	0.6 cc.	0.8 cc.
Hemolysis.....	+	P	–	–	–	–	+	+	+	+	+	+	+	+

P indicates partial reaction.

The titer is 0.03, and no anticomplementary action is exerted in any of the dilutions.

The above results show the importance of selecting the proper peptone for the production of antigen. The Fairchild peptone possesses very pronounced advantages over the other two in this particular instance of antigen production; that is for the complement fixation test for infectious abortion. Other American brands which have not been used in this comparative study may be equally satisfactory, however.

The growths obtained on Fairchild peptone agar were quite luxuriant as compared with those of the other brands employed. The suspensions prepared directly from the agar slants were so dense that it was impossible to make turbidity determinations with the nephelometer without diluting at least five times. A further dilution of 1:5 was necessary before making the antigen titration. The titer of this antigen remained constant for at least seven months. The results obtained with Fairchild peptone have been duplicated again and again, and by following the method of antigen production as revised or elaborated by us no difficulty has been experienced in the preparation of antigen for complement fixation work in connection with infectious abortion.

Solutions of Fairchild peptone are decidedly acid, and much care must be exercised in properly neutralizing agar that is prepared with this peptone. A final hydrogen ion concentration of P_H 6.8 appears to be the most favorable. The composition of the agar medium as we have been employing it for some time is as follows:

Water	1000 cc.
Meat extract (Liebig)	4 grams, or 0.04 per cent
Fairchild peptone	10 grams, or 1.0 per cent
Agar, granular	16 grams, or 1.6 per cent

3. Antigen production by different strains of B. abortus

Seven different strains of *B. abortus* were employed in these experiments. One was an old stock culture which was obtained from the University of Wisconsin, and labeled "Bang." It had come originally from Denmark. The others were of different ages varying from a few months to five years. Two of the strains still refused to grow by the ordinary aerobic method. Two of the newer strains had been isolated by the writers from the intestine of prematurely-born calves of infected dams. The hydrogen ion concentration of the medium (ordinary peptone agar) was P_H 6.8.

Good growths were obtained on all of the tubes when Fairchild agar was employed, though there was a decided difference in favor of the older and of the aerobic strains. On agar containing the other two American brands of peptone previously mentioned (A and B) the growths were less luxuriant in the aerobic cultures, and scant in the anaerobic. However, all of the suspensions prepared from the readily-visible growths exercised antigenic properties when subjected to the titration tests. The most satisfactory results were obtained with the Fairchild agar antigens. The antigen titer for the seven different strains was fairly uniform here, varying only between 0.03 and 0.05.

The conclusion may be drawn, therefore, that the different strains of *B. abortus* have antigen-producing powers, and that it matters little whether one or another of the organisms is used for this purpose, providing sufficient growth is produced within the desired period of incubation (four to five days). Largely because of the ease with which abundant antigen may be obtained, the Bang strain has been used almost exclusively by the writers. The advantages of employing polyvalent antigen may be such that it will be advisable to make use of at least three or four representative strains, instead of one. This we are doing at the present time.

4. Determination of the most favorable hydrogen ion concentration for B. abortus antigen production

It soon became quite evident, in the course of this work, that the hydrogen ion concentration of the agar medium upon which the organism is grown is a very important factor. *B. abortus* has the property of changing the initial hydrogen ion concentration of the medium over quite a range and in either direction from the neutral point, to suit its own need, as Evans (1918) has shown before us. It seems quite apparent, then, that this organism requires an optimum concentration for its best development. By the colorometric method of exact determination of Clark and Lubs this optimum range can be definitely established. From the results of preliminary experiments it appeared as if the most favorable concentration was at or near P_H 6.8. Consequently this point was taken as a mean in subsequent experiments. All of the cultures were grown in tubes of Fairchild peptone agar of definite but different H ion concentration, and the antigens carefully titrated. A number of interesting facts were brought out.

First, it appears that, if the initial hydrogen ion concentration is P_H 6.8, the reaction does not change during the growth of the culture, and that antigens prepared from such growths are more satisfactory than those obtained from culture tubes on which the organisms themselves readjust the hydrogen ion concentration during the course of incubation. If the initial H ion concentration of the agar is at any other point between P_H 6.6 and P_H 7.3 growth is slow until the optimum concentration P_H 6.8 is reached. Unless this readjustment is very slow, as we have found it to be when anaerobic strains of *B. abortus* are used, normal growth takes place. Some time is consumed however, at best, and there is some loss of antigen; furthermore, if the usual incubation period is exceeded, there will be danger of anticomplementary action when the antigen is employed in the regular fixation test. Because of their greater ability to adjust the reaction of the medium within the limits of the P_H 6.6 and P_H 7.3 range, the aerobic strains are better adapted for antigen production than the anaerobic, irrespective of any other advantages or disadvantages which they might possess. By anaerobic strains are meant, of course, those strains of *B. abortus* which require partial exclusion of oxygen for their development.

ADJUSTMENT OF HYDROGEN ION CONCENTRATION, IN THE PREPARATION OF FAIRCHILD PEPTONE AGAR FOR ANTIGEN PRODUCTION

Saturated solution of sodium carbonate is employed for the neutralization of the medium. Instead of adjusting the reaction at once to P_H 6.8, the optimum for good antigen production, the point 6.5 is sought. By careful experimentation we have found that if the initial hydrogen ion concentration, that is just before sterilization, is reduced to P_H 6.5, the final concentration after sterilization for fifteen minutes under 15 pounds of extra pressure is ordinarily P_H 6.8—the point desired. These observations apply to nutrient agar containing Fairchild peptone, which is quite acid, and when sodium carbonate is used as the neutralizing agent. Aside from the use of Fairchild peptone, and of the above procedure for regulating the acidity, the method of preparing the agar is the same as in our daily laboratory routine.

TITRATION OF THE ANTIGEN

By the use of the McFarland nephelometer we have been enabled greatly to simplify and standardize certain steps which are preliminary to the actual titration. Heretofore no exact method of dilution of the bacterial suspension was followed. As a result titrations frequently had to be repeated with different dilutions of antigen until the final dilution was reached within which the limits of the titration scheme fell.

We have found it desirable to prepare bacterial suspensions which are quite dense; that is by washing off the slant agar growths of *B. abortus* with relatively small amounts of the saline solution. From the concentrated stock suspension thus prepared a dilution is made with carbolized saline solution to match tube 1.75 of the nephelometer set. This diluted antigen will, as a rule, furnish the final titration figure in a single antigen titration. By knowing the exact proportion in which the antigen was diluted, it is only necessary after the first and only titration to prepare the proper dilution of antigen from the stock suspension, without further use of the nephelometer set. This method has been employed in all of the work of the past two years and has proven itself reliable and time-saving.

The Zinsser (1918) method of preserving antigen has been employed by the writers, with gratifying results. This differs from the usual method in that the bacterial growths on slant agar are washed off with 10 to 17 per cent saline solution, instead of 0.85, and diluting this stock suspension with distilled water to 0.85 per cent sodium chloride content when needed. This method has been of particular advantage during the warm summer months and under conditions of imperfect refrigeration. We have had little difficulty, however, in preserving antigen by the usual method over periods of at least five or six months.

OTHER MODIFICATIONS OF TECHNIC INVOLVED IN THE COMPLEMENT FIXATION TEST

Titration of immune serum

In the titration of the antigen an immune serum of known strength is required. Instead of conducting several fixation tests with various dilutions of the cow's serum, as has been customary heretofore, one test can be made to suffice by employing all of the serum dilutions at one and the same time, as is shown in the following titration scheme.

Titration of immune serum

TUBES	NaCl 0.85 PER CENT	IMMUNE SERUM	COMPLEMENT TITER 0.03	ANTIGEN TITER 0.03	TIME	HEMOLYSIN TITER 0.03	SHEEP'S CORPUSCLES 2 PER CENT	TIME	RESULTS
	cc.	*cc.*	*cc.*	*cc.*		*cc.*	*cc.*		
1	1.5	0.01	0.045	0.12	Incubate 1 hour at 37°C.	0.15	0.5	Incubate 2 hours at 37°C.	Complete hemolysis
2	1.5	0.02	0.045	0.12		0.15	0.5		Partial hemolysis
3	1.5	0.03	0.045	0.12		0.15	0.5		No hemolysis
4	1.5	0.04	0.045	0.12		0.15	0.5		No hemolysis
5	1.5	0.05	0.045	0.12		0.15	0.5		No hemolysis
6	1.5	None	0.045	0.12		0.15	0.5		Complete hemolysis

The titer of the immune serum is 0.03 cc. This amount of the serum is added to each of the first six tubes in the antigen titration.

The use of positive and negative sera as controls

The descriptions of methods for conducting the complement fixation test in infectious abortion, which have come to our attention, have not provided for the use of special control sera. The inclusion of a positive and negative control serum is of much importance. Control sera may be obtained readily from animals whose reactions have previously been determined, and, if carbolized when fresh with 0.5 per cent phenol, they will keep for at least six months. The positive and the negative serum are used in the same manner as the test samples, and at the same time.

SUMMARY

The complement fixation test as applied to infectious abortion is specific, and serves as a valuable method of diagnosis.

With the partial revision and simplification of technic presented in this paper the method should be thoroughly practical and reliable.

The most satisfactory antigen was obtained on nutrient agar containing Fairchild peptone, and having an initial hydrogen ion concentration of P_H 6.8.

The incubation period of the *B. abortus* cultures should not exceed four to five days, and the stock antigen suspensions should be prepared immediately following the removal of the culture tubes from the incubator.

Antigen suspensions with a turbidity of 1.75 in terms of the McFarland nephelometer lend themselves readily for direct antigen titration.

The Wenner method of bleeding guinea-pigs, with the present refinement, is a very practical and economic one, and can be mastered readily by the ordinary operator.

Complement stabilized with 40 per cent of a 12 per cent sodium acetate solution retains its complementary properties for three to four weeks.

Formalinized sheep's blood may be used for three to four weeks as the immediate source of hemolytic antigen in the fixation test. Freshly-washed corpuscles from formalinized blood may be used also as antigen for hemolysin production.

Positive and negative sera should be used as controls in the final fixation tests.

REFERENCES

Bang, B.: Die Aetiologie des Seuchenhaften ("infectiösen") Verwerfens. Zeitschr. Tiermed., 1897, **1**, 241–278.

Bernstein, E. P., and Kaliski, D. J.: The use of formalinized sheep cells in complement fixation tests. Zeitschr. Immun., Orig., 1912, **13**, 490–495.

Clark, Wm. M., and Lubs, H. A.: The colorimetric determination of hydrogen ion concentration and its applications in bacteriology. Jour. Bact., 1917, **2**, 1–34, 109–136, and 191–236.

Coca, A. F.: A rapid and efficient method of producing hemolytic amboceptor against sheep corpuscles. J. Inf. Dis., 1915, **17**, 361–398.

Evans, Alice C.: Further studies on *Bacterium abortus* and related bacteria. J. Inf. Dis., 1918, **22**, 576–593.

Hadley, F. B., and Beach, B. A.: The diagnosis of contagious abortion in cattle by means of the complement fixation test. Wisc. University Agricultural Experiment Research Bulletin, June, 1912, no. 24.

Holth, H.: Die agglutination und die Komplementbindungs-Methode in der Diagnose des seuchenhaften Verwerfens der Kühe. Berlin. tierärtzl. Woch., 1909, **25**, 686–687.

McFarland, J.: The nephelometer. J. Am. Med. Assoc., 1907, **49**, 1176–1178.

Mohler, J. R., and Traum, J.: Infectious abortion of cattle. 28th Ann. Rep. Bur. An. Ind., U. S. Dept. Agr., 1911, 147–183.

Rettger, L. F., and White, G. C.: Infectious abortion in cattle. Storrs Agr. Exp. Sta. Bulletin 93, Jan. 1918.

Rhamy, B. W.: Further studies on the preservation of complement by sodium acetate. J. Am. Med. Assoc., 1918, **70**, 2000–2001.

Stitt, E. R.: A method of producing rabbit's hemolytic amboceptor. Practical Bacteriology, Blood Work and Parasitology, 1918, 200.

Surface, F. M.: The diagnosis of infectious abortion in cattle. Ky. Agr. Exp. Sta. Bulletin 166, June, 1912, 303–366.

Wall, Sven: Ueber die Festellung des seuchenhaften Abortus beim Rinde durch Agglutination und Komplementbindung. Zeitschr. Infektionskr., 1911, **10**, 23–55.

Wenner, J. J.: A note on bleeding guinea-pigs and on preserving sheep's erythrocytes. J. Immun., 1918, **3**, 389–393.

Zinsser, H.: Infection and Resistance. 2d edition, 1918, p. 71.

THE ANTIGENIC PROPERTIES OF GLOBIN, WITH A NOTE ON THE INDEPENDENCE OF THE PROPERTIES OF SERUM AND TISSUE PROTEINS, AS EXEMPLIFIED BY THE ABSENCE OF ANTIBODY FROM THE GLOBIN OF AN IMMUNISED ANIMAL[1]

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In a previous paper (4) we described experiments conducted with the serum of rabbits which had received repeated injections of globin, the protein constituent of hemoglobin, derived from guinea-pig's blood. The results showed that globin could act as an antigen and that, together with the corresponding antiserum, fixation of complement was produced. A high degree of complement-fixation was obtained with seven different specimens of guinea-pig's globin along with one or the other of two antisera. The striking character of the reaction is shown by the fact that with a suitable specimen of guinea-pig's complement, after incubation with 0.5 cc. 1 per cent globin solution plus 0.05 cc. of antiserum (55°C.) for one and one-half hours, more than 60 doses of complement were required in order to hemolyse the test corpuscles, whereas 1 dose of complement gave complete lysis in the presence of the same amount of globin alone, and 2½ doses gave complete lysis along with the antiserum in saline. (The minimal hemolytic dose of complement for 1 cc. ox corpuscles plus 5 minimal hemolytic doses of immune body from the rabbit was 0.01 cc.) Globin when mixed with serum causes a precipitate, but this precipitate *per se* does not lead to complement-fixation. Thus globin failed to fix complement along with normal rabbit serum or with a variety of heterologous antisera from the rabbit.

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[1] We have pleasure in acknowledging a grant from the Carnegie Trust toward the expenses of this work.

In view of the suggestion that protein contamination was responsible for the development of the antisera which we obtained, the following observations are worthy of note: (1) the solution of globin gave no fixation of complement with anti-human serum; (2) the antiglobin serum did not fix complement with guinea-pig's serum or acid serum-albumin; (3) anti-guinea-pig globin did not contain hemolytic immune body for guinea-pig's red corpuscles. Thus the most likely contaminations—from human protein due to handling, from imperfect removal of serum from the guinea-pig's red corpuscles or from receptors of the red corpuscles which might have passed through the filter (as Muir and Ferguson (6) observed)—were all excluded. The present communication contains further work on the subject, which was interrupted owing to the difficulties described later, but which it appears advisable to publish, especially in view of the fact that our main conclusion, that globin possesses antigenic properties, has been questioned and the result, which we obtained, has been attributed to contamination (Gay and Robertson (5, 7)).

PREPARATION OF GLOBIN

The method employed was based on that of Schulz (11), which depends on the fact that when hemoglobin has been dissociated by a suitable concentration of HCl in watery medium, the acid hematin can be extracted in great part by ether and alcohol. In regard to our preparations of globin, it is to be noted that (1) the blood corpuscles were, to begin with, repeatedly washed with the centrifuge in order to remove any trace of serum, (2) the laked blood was first centrifuged and then filtered through Berkefeld or Maassen candles to remove stromata prior to treatment with acid. After extraction of the pigment, excess of acid was removed by dialysis and the ether was evaporated off by warming. The details have already been fully described (4). The globin solution equivalent to 1 per cent of washed blood sediment is referred to throughout this work as "1 per cent" globin.

The solution of guinea-pig globin which has been subjected to prolonged dialysis against distilled water is highly sensitive toward salt, forming a precipitate in 0.85 per cent NaCl solution. It is also precipitated from distilled water by traces of alkali; thus N/20,000 NaOH caused clouding of the 1 per cent solution after warming at 37°C. for several hours. The addition of N/2,500 HCl to the salt solution prevented precipitation, but N/5,000 acid did not suffice; on the other hand, when precipitate had formed, the addition of N/2,500 HCl did not cause it to dissolve completely. Thus the preparation possessed the characters described by Schulz. As a rule, solutions were employed which had been dialysed for a shorter time (twenty-four hours against soft tap water) and which, although reacting neutral to litmus paper, retained sufficient acid to remain clear in the presence of 0.85 per cent NaCl.

In addition to the two specimens of antiserum to guinea-pig's globin previously described, two antisera, also from rabbits, were prepared similarly against ox globin.

FACTORS WHICH INFLUENCE COMPLEMENT-FIXATION BY GLOBIN PLUS ANTIGLOBIN SERUM

It was found that the complement-fixation reaction was influenced to a marked degree by physico-chemical factors; namely, (a) the relative proportions of antigen to antibody (zone phenomenon), (b) presence of acid or alkali (hydrogen-ion concentration), as well as by the individual properties of the complement-containing serum and of the animal receiving the injections of globin.

1. *Quantitative relationships between antigen and antibody*

The following is a characteristic example:

Ox globin (dialysed solution which gave no precipitate in 0.85 per cent NaCl solution); three series were made—A, containing 1 cc. 0.33 per cent globin; B, containing 1 cc. 1.0 per cent globin; C, containing 1 cc. 2.0 per cent globin—each tube contained 0.05 cc. antiglobin serum; ascending amounts of complement (guinea-pig serum) were then added and the mixtures incubated for one and a half hours at 37°C.

Finally the test corpuscles were added (consisting of 1 cc. 3 per cent washed ox blood suspension plus 5 minimal hemolytic doses of immune body from the rabbit in all the experiments unless otherwise specified) and there was a further period of incubation for one and a quarter hours. The readings were made, as in all such experiments, after the tubes had stood overnight at room-temperature. The results were as follows:

SERIES	LYSIS WITH THE FOLLOWING AMOUNTS OF COMPLEMENT			
	0.06 cc.	0.09 cc.	0.13 cc.	0.18 cc.
A	None	None	None	Distinct
B	None	Distinct	Marked	Marked
C	Marked	Marked	Very marked	Just complete

Controls: Antiserum 0.05 cc. alone in 1 cc. saline plus 0.025 cc. of complement = complete lysis.

Globin solutions alone plus 0.01 cc. complement = complete lysis.

On the other hand, when the amount of antiserum was varied a point was reached any diminution below which caused a very great falling off in the amount of complement fixed, as the following experiment shows:

Guinea-pig globin solution 0.5 cc. plus antiglobin serum in series (A) 0.05 cc., in (B) 0.025 cc., and in (C) 0.01 cc.

SERIES	LYSIS WITH THE FOLLOWING AMOUNTS OF COMPLEMENT				
	0.025 cc.	0.035 cc.	0.075 cc.	0.1 cc.	0.15 cc.
A	None	None	None	Trace	Incomplete
B	Almost complete	Complete	Complete	Complete	Complete
C	Almost complete	Complete	Complete	Complete	Complete

Controls: Antiserum 0.05 cc. plus 0.5 cc. saline plus 0.015 cc. of complement = complete lysis.

Globin solution 0.5 cc. plus 0.015 cc .of complement = complete lysis.

2. *Hydrogen-ion concentration*

The results previously published showed clearly that the amount of complement which was fixed by guinea-pig globin plus antiglobin serum depended on the hydrogen-ion concentration, and it appeared as if the optimum lay slightly to the acid side of neutral, just as Sachs and Altmann (8) had found in the case of the Wassermann syphilis reaction. Thus in the case of a specimen of globin, which had been dialysed for a week against distilled water and which was highly sensitive to the precipitating action of 0.85 per cent NaCl, the addition of N / 2,500 HCl more than doubled the amount of complement which was fixed by antigen plus antibody. On the other hand, it appeared that too high a degree of acidity diminished the amount of complement fixed, as the addition of alkali to such acid solutions increased the complement-fixation. The question of hydrogen-ion concentration apparently has not been fully taken into consideration by Gay and Robertson and Schmidt (9) as a possible explanation of the failure to obtain complement-fixation in their experiments.

The hydrogen-ion concentration, or the physical changes which resulted therefrom, was probably also the determining factor in the following experiment:

Guinea-pig globin (preparation V)—a 13 per cent stock solution which when diluted to 0.9 per cent remained clear in 0.85 per cent saline (= untreated globin): 10 cc. of this stock solution were precipitated by saturation with NaCl; the precipitate was removed by centrifuging and was then dialysed against running water for two days, and finally diluted to 0.9 per cent (= salted globin). In the usual complement-fixation tests 0.5 cc. of *untreated* globin plus 0.05 cc. of antiserum plus 0.15 cc. of guinea-pig's complement gave a trace of lysis of the test corpuscles: 0.5 cc. of salted globin plus 0.05 cc. of antiserum plus 0.04 cc. complement gave complete lysis of the test corpuscles.

Control: Each globin solution alone plus 0.01 cc. complement gave complete lysis.

3. *The individual property of the complement-containing serum*

The importance of individual properties of the complement-containing serum in determining the amount of complement which is fixed, has been pointed out by Browning and Mackenzie (2) and Browning and Kennaway (1) in the case of the Wassermann syphilis reaction and it has been shown that there is no constant relationship between the hemolytic power and the deviability of the complement. It was shown also that by dialysing complement the hemolytic power might remain almost unaltered, while the deviability was greatly reduced (Browning and Mackie (3)). Similar variations in deviability were found by us (4) in the case of fixation by globin plus antiglobin serum. In addition, we have tested mixtures of guinea-pig globin plus antiserum, which actively fixed guinea-pig's complement, using (a) rabbit's complement and testing with the usual sensitised ox blood suspension and (b) ox complement, with guinea-pig's corpuscles as the indicator, the immune body in the latter case being that naturally present in the ox serum. In neither instance could complement-fixation be detected.

4. *The individual character of the animal which receives the injections of antigen*

The methods which were used to develop antisera have been previously described in detail and consisted in repeated intraperitoneal injections of globin in suspension or solution, or intravenous injections of solutions. The antisera to guinea-pig's globin were derived from the second and third animals injected. The first animal apparently failed to yield demonstrable antibody, but, as we were at the time unaware of the great importance attaching to the relative proportions of antigen and antibody and of the optimum reaction, the antibody may have been missed. Similarly, the antisera to ox globin were derived from the second and third animals tested. But later, repeated attempts to obtain further antisera failed both in the case of ox and of guinea-pig globin and in spite of the knowledge gained as to the conditions for eliciting the reaction *in vitro*. Thus we are forced to the conclusion that globin is not a potent antigen or, in Ehrlich's language, that it does not produce a powerful "ictus immunisatorius" except in specially suitable individual animals. Of course, the factor of variable responsiveness is a commonplace experience in the practice of immunisation; but this may well explain why Gay and Robertson (5) and Schmidt (9) failed to obtain an antiserum to globin from the three rabbits which they tested. Similarly, in view of our experiences, the development of antisera which fixed complement in the presence of globin alone, as the result of injecting a compound of globin with casein into two rabbits, may have been fortuitous rather than due to any special effect of the casein, as explained by Gay and Robertson. In any case the statements of these workers that (1) globin is not an antigen but that (2) injections of globin-casein cause the development of antisera which lead to complement-fixation with a solution of pure globin, would appear almost to involve a contradiction in terms.

THE BEHAVIOR OF HEMOGLOBIN AS AN ANTIGEN

As was shown by us previously, an antiserum which reacted intensely with globin (faintly acid solution, clear in 0.85 per cent NaCl) failed to give practically any fixation of complement with the same specimen of hemoglobin as that from which the globin was derived, and in the corresponding concentration. The following is a further illustration:

Guinea-pig's globin solution 0.5 cc. plus 0.05 cc. antiglobin plus 0.3 cc. complement = trace of lysis of test corpuscles. 0.5 cc. corresponding solution of hemoglobin plus 0.05 cc. antiglobin plus 0.04 cc. complement = just complete lysis.

Controls: Globin and hemoglobin solutions alone plus 0.01 cc. complement = just complete lysis.

0.05 cc. antiglobin serum in 0.5 cc. saline + 0.03 cc. complement = just complete lysis.

Thus neutral hemoglobin does not react with a potent antiglobin serum. The failure of hemoglobin to react with the antiglobin serum appears to us to afford strong evidence in favor of the antibody which we demonstrated being in reality developed by the globin and not by some adventitious constituent, as suggested by Gay and Robertson. In view of what has been stated already the only possible contamination would appear to be bacterial; now it was the solution of hemoglobin which was especially exposed to infection. During the subsequent procedure, involving the addition of acid, alcohol and ether, conditions were much more unfavorable for contamination and, as has been noted, the stock solutions of globin remained perfectly clear for many weeks. Attempts to produce antibodies to hemoglobin appear to have been generally, but not universally unsuccessful (see Schmidt and Bennett (10) for an extensive review of the literature as well as original experiments). Our own attempts to produce antisera to guinea-pig's hemoglobin in rabbits were likewise mainly unsuccessful and the serum also contained no lytic immune body for guinea-pig's red corpuscles, as tested with rabbit's complement. But in one of our experiments marked complement-fixation was obtained with several specimens of guinea-pig's hemoglobin along with the heated serum of a rabbit which had received several injections of guinea-pig's hemoglobin. In view of the small number of our observations and the fact that hemoglobin was necessarily tested in neutral solution, we hesitate to deny that hemoglobin altogether lacks antigenic properties; but the proportion of animals which react, as shown by the production of complement-fixing antibody, is small, and it is evident that any antigenic power which it may possess is very weak. Schmidt and Bennett's failure to demonstrate antisubstance in the serum of eight rabbits after repeated injections of hemoglobin is in agreement with this conclusion.

THE TOXICITY OF GLOBIN AND HEMOGLOBIN

Gay and Robertson found that their specimens of globin were toxic, especially for guinea-pigs which apparently received an intravenous or intraperitoneal injection of an acid solution. The combination of globin with casein was stated to be non-toxic, but "marked symptoms of prostration with polypnoea after each injection" are mentioned as occurring in one of the two rabbits which they injected with globin-casein. Our specimens of globin caused no obvious ill effects in rabbits. Hemoglobin was found to be non-toxic in our experiments as well as in those of Schmidt and Bennett and of others.

THE SPECIES-SPECIFICITY OF GLOBIN

In our previous work the action of an antiserum to guinea-pig's globin was tested in parallel series with guinea-pig's globin and with preparations from ox and rabbit blood. In each case the specimen of globin was thoroughly dialysed and N/2,000 HCl added and the same concentration of globin and amount of antiserum were employed in each case. The result was that, as compared with the amount of complement fixed with homologous globin (taken as 100 per cent), rabbit globin caused the absorption of 16 per cent of complement and ox globin of less than 6 per cent. Thus a marked degree of species-specificity was shown to exist. However, when globin from further species was tested along with antisera both for guinea-pig and for ox globin results were obtained which are highly complex and difficult to interpret, as the following examples show:

A. Complement-fixation produced by mixtures of anti-ox globin serum 0.025 cc. plus 0.5 cc. of different species of globin in 0.2 per cent solution (tested with 0.5 cc. sensitised ox corpuscles: minimal hemolytic dose of complement = 0.015 cc.)

SPECIES OF GLOBIN	LYSIS OF 0.5 CC. TEST CORPUSCLES WITH THE FOLLOWING AMOUNTS OF GUINEA-PIG'S COMPLEMENT		
	0.025 cc.	0.04 cc.	0.065 cc.
Ox	Faint trace	Faint trace	Faint trace
Goat	None	None	None
Duck	None	None	Faint trace
Rabbit	Almost complete	Complete	Complete

Control: Globin solutions alone plus 0.015 cc. complement = complete lysis in every case.

Antiserum 0.025 cc. in saline plus 0.02 cc. complement = complete lysis.

B. Guinea-pig and ox globin tested for complement-fixation in parallel and in crossed series with the respective antisera.

0.5 CC. GLOBIN (0.33 PER CENT SOLUTIONS)	ANTISERUM 0.05 CC.	AMOUNTS OF GUINEA-PIG'S COMPLEMENT				
		0.05 cc.	0.075 cc.	0.1 cc.	0.13 cc.	0.18 cc.
Guinea-pig	Anti-guinea pig	—	Trace	Distinct	Marked	Very marked
Guinea-pig	Anti-ox	—	Trace	Marked	Marked	Almost complete
Ox	Anti-ox	—	Trace	Trace	Distinct	Marked
Ox	Anti-guinea pig	Just complete	Complete	Complete	Complete	Complete

Controls: Globin solutions plus 0.01 cc. complement = complete lysis.

Anti-guinea-pig globin in saline plus 0.04 cc. complement = very marked lysis.

Anti-ox globin in saline plus 0.05 cc. complement = very marked lysis.

Thus anti-ox globin fixes complement actively with goat and duck globin. Anti-ox globin also fixes complement along with guinea-pig's globin; on the other hand, as we found previously, anti-guinea-pig globin fixes practically no complement along with ox globin. Any fallacy would appear to be excluded from the latter results of "crossed" experiments by the fact that both specimens of globin were tested with the homologous and the heterologous antisera at the same time and with the same complement. In both homologous series marked fixation of complement was observed, thus showing that the reagents were acting satisfactorily. Again, anti-ox globin fixes little or no complement in the presence of rabbit globin. It was hoped to develop the interesting lines of work suggested by these results, but this had to be given up owing to the continued failure to obtain further antisera. Thus while evidence of species-specificity exists in certain cases, there is also a wide, though not universal, community of antigenic properties shared by the globin of widely separate animal species. It will be difficult to explain these results on any basis of hypothetical contamination.

SUMMARY

1. Globin can act as an antigen. In addition to two antisera for guinea-pig's globin, two antisera for ox globin have also been obtained from rabbits. With these antisera and a number of different specimens of the homologous globins powerful complement-fixation reactions have been obtained. The obtaining of the reaction depends on suitable quantitative relationships between antigen and antibody and also, as was shown previously, on a suitable hydrogen-ion concentration.

2. Only certain rabbits apparently respond to injections of globin by the production of complement-fixing antibodies. The injections of globin caused no obvious toxic effects in rabbits. Hemoglobin seems to elicit antibody production more rarely.

3. The reactions with antisera show, in certain cases, marked species-specificity of globin; thus the antiserum to guinea-pig's globin does not fix complement with ox globin. On the other hand, anti-ox globin fixes complement along with goat, duck and guinea-pig globin but not with rabbit globin; no explanation is offered of the contradictory behavior of ox and guinea-pig globins in the crossed experiments. But the results taken together seem to exclude bacterial contamination as the cause.

4. The evidence points to the phenomena being due to a genuine antibody to globin and not to adventitious protein contamination. Further recorded facts (namely, that anti-guinea-pig globin does not contain hemolytic immune body for guinea-pig corpuscles and does not react with guinea-pig's hemoglobin, serum or acid serum-albumin, and that guinea-pig globin does not react with antihuman serum) also exclude the probable contaminations which might arise in the course of preparation of the globin solutions.

NOTE ON THE INDEPENDENCE OF THE PROPERTIES OF SERUM AND TISSUE PROTEINS, AS EXEMPLIFIED BY THE ABSENCE OF ANTIBODY FROM THE GLOBIN OF AN IMMUNISED ANIMAL

An example of the independence of properties of tissue and of serum proteins was obtained in the following experiment. Globin was prepared from the hemoglobin of a rabbit which had been repeatedly injected with washed ox blood and which in consequence contained abundance of the corresponding hemolytic immune body in its serum (minimal hemolytic dose of immune body for 1 cc. 3 per cent ox blood suspension *plus* excess of guinea-pig's complement = 0.0025 cc.). Amounts of the globin up to 0.5 cc. of a 6.5 per cent solution (both in saline and in saline plus N/2,000 HCl, which barely sufficed to keep the globin completely in solution) led to no hemolysis of 1 cc. of 3 per cent ox blood suspension in the presence of 0.1 cc. guinea-pig's complement. Thus antibody was absent from the globin of an animal which had reacted to an antigen by the development of powerful serum antibodies.

REFERENCES

(1) Browning and Kennaway: Lancet, 1919, **1**, 785.
(2) Browning and Mackenzie: Zeitschr. f. Immunitätsforsch., Orig., 1909, **2**, 459.
(3) Browning and Mackie: Zeitschr. f. Immunitätsforsch., Orig., 1914, **21**, 422.
(4) Browning and Wilson: Jour. Path. and Bact., 1909, **14**, 174.
(5) Gay and Robertson: Jour. Exper. Med., 1913, **17**, 535.
(6) Muir and Ferguson: Jour. Path. and Bact., 1906, **11**, 84; see also Muir's Studies on Immunity, London, 1909.
(7) Robertson: The Physical Chemistry of the Proteins. New York and London, 1918, p. 155.
(8) Sachs and Altmann: Berl. klin. Woch., 1908, **45**, 699.
(9) Schmidt: University of California Publications in Pathology, 1916, **2**, 182.
(10) Schmidt and Bennett: Jour. Infect. Dis., 1919, **25**, 207.
(11) Schulz: Ztschr. f. Physiol. Chem., 1898, **24**, 449.

THE PROTECTIVE VALUE OF PNEUMOCOCCUS VACCINATION IN MICE AND RABBITS[1]

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Although the fact is well known that inoculation of animals with the pneumococcus develops against subsequent inoculation with virulent cultures an immunity which is specific for the homologous type of pneumococcus, there is no record of a quantitative determination of the degree of immunity that is obtained after vaccination with standardized vaccine. Accordingly a series of experiments with the inoculation of mice with pneumococcus vaccines of types I, II, and III were made, the results of which it is the purpose of this paper to record.

Miss Thelma L. Franklin, of the laboratory staff, prepared vaccines of pneumococcus types I, II, and III according to the standard methods used in the laboratory and vaccinated mice with these vaccines. In order to test the protection obtained as a result of the vaccination, living, virulent cultures were inoculated seven days after the last dose of the vaccine. Standard cultures of pneumococci of types I, II, and III are maintained in the laboratory at a virulence which fluctuates considerably from time to time but which usually kills mice of eighteen grams weight in a dose of 0.000001 cc. in less than thirty-six hours. The variation in virulence in the normal or control mice is well shown in the tables recording the results of the experiments. On account of this fluctuation in virulence it is not practical in experimentation with the pneumococcus to determine

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Experiment I (4-18-19). Mice receiving one inoculation of 1 cc. of type I vaccine, containing three billion organisms per cubic centimeter; tested seven days after inoculation with virulent broth cultures of types I, II, and III

CULTURE—PNEUMOCOCCUS I					CULTURE—PNEUMOCOCCUS II					CULTURE—PNEUMOCOCCUS III				
Quantity	Number of mice inoculated	Number of mice lived	Number of mice died	Time animal died	Quantity	Number of mice inoculated	Number of mice lived	Number of mice died	Time animal died	Quantity	Number of mice inoculated	Number of mice lived	Number of mice died	Time animal died
cc.				*hours*	*cc.*				*hours*	*cc.*				*hours*
0.000001	3	1	2	46	0.000001	3	0	3	23–44	0.000001	3	0	3	28–47
0.00001	3	1	2	36	0.00001	3	0	3	23–38	0.00001	3	0	3	17–22
0.0001	3	0	3	24–31	0.0001	3	0	3	17–35	0.0001	3	0	3	16
Control—Normal mouse					Control—Normal mouse					Control—Normal mouse				
0.000001	1		1	32	0.000001	1		1	29	0.000001	1		1	33

Experiment II (5-14-19). Mice receiving three weekly inoculations of 1 cc. of type II vaccine, containing three billion organisms per cubic centimeter; tested seven days after last inoculation with virulent broth cultures of Types I, II, and III

0.00001	3	0	3	28–36	0.00001	3	2	1	52	0.00001	3	0	3	29–38
0.0001	3	0	3	28–32	0.0001	3	1	2	27–34	0.0001	2	0	2	22–34
0.001	2	0	2	28–35	0.001	3	0	3	34–38	0.001	2	0	2	26–30
Controls—Normal mice					Controls—Normal mice					Controls—Normal mice				
0.000001	1		1	47	0.000001	1		1	–24	0.000001	1		1	–41
0.00001	1		1	40	0.00001	1		1	–39	0.00001	1		1	–45
0.0001	1		1	29	0.0001	1		1	–29	0.0001	1		1	–45

Experiment III (6-10-19). Mice receiving six weekly inoculations of 1 cc. of type III vaccine, containing three billion organisms per cubic centimeter; tested seven days after last inoculation with virulent broth cultures of Types I, II, and III

0.000001	3	0	3	24–35	0.000001	3	0	3	16–25	0.00001	3	0	3	27–50
0.00001	3	0	3	20–28	0.00001	3	0	3	26–28	0.0001	3	0	3	19–27–84
0.0001	2	0	2	28–35	0.0001	2	0	2	28–32	0.001	3	0	3	26–27
Controls—Normal mice					Controls—Normal mice					Controls—Normal mice				
0.000001	1		1	32	0.000001	1		1	−36	0.000001	1		1	34
0.00001	1		1	−29	0.00001	1		1	−25	0.00001	1		1	−35

Experiment IV (6-9-19). Mice receiving three weekly inoculations of 1 cc. of type I vaccine, containing three billion organisms per cubic centimeter; tested seven days after last inoculation with virulent broth cultures of Types I, II, and III

0.000001	3	3	0		0.00001	3	0	3	28–40	0.00001	3	0	3	−23–51
0.00001	3	3	0		0.0001	3	0	3	22–40	0.0001	2	0	2	−30–51
0.0001	3	3	0		0.001	2	0	2	22–29	0.001	2	0	2	−23
0.001	3	0	3	21–37										
Controls—Normal mice					Controls—Normal mice					Controls—Normal mice				
0.000001	1		1	−36	0.000001	1		1	−38	0.000001	1		1	−33
0.00001	1		1	−29	0.00001	1		1	−37	0.00001	1		1	−30
0.0001	1		1	−27										

Experiment V (7-10-19). Mice receiving 6 inoculations at six-day intervals of 0.5 cc. of type I vaccine or six billion cocci in each dose, and tested seven days after last inoculation

CULTURE—PNEUMOCOCCUS I					CULTURE—PNEUMOCOCCUS II					CULTURE—PNEUMOCOCCUS III				
Quantity	Number of mice inoculated	Number of mice lived	Number of mice died	Time animal died	Quantity	Number of mice inoculated	Number of mice lived	Number of mice died	Time animal died	Quantity	Number of mice inoculated	Number of mice lived	Number of mice died	Time animal died
cc.				*hours*	*cc.*				*hours*	*cc.*				*hours*
0.0001	2	1	1	37	0.00002	2	0	2	24–36	0.00002	2	0	2	25–50
0.001	2	2	0	14 days										
Control—Normal mice					Control—Normal mice					Control—Normal mice				
0.000001	1		1	–38	0.000001	1		1	–36	0.000001	1		1	–43
0.0001	1		1	–25	0.00002	1		1	–32	0.00002	1		1	–34

Experiment VI (7-31-19). Mice receiving six inoculations at six-day intervals of 0.5 cc. of type I vaccine or six billion cocci in each dose, and tested four weeks after the last inoculation

CULTURE PNEUMOCOCCUS I				
Quantity	Number of mice inoculated	Number of mice lived	Number of mice died	Time animal died
cc.				*hours*
0.01	2	0	2	–22
0.1	2	0	2	–22
0.5	2	0	2	–22
Control—Normal mouse				
0.000001	1		1	–28

too exactly the minimal lethal dose, but it is practical to maintain this standard. The dosage of the test virulent culture, therefore, was estimated in multiples of this dose.

Preparation of the vaccine. Meat infusion peptone broth was the medium used with 0.5 per cent glucose. Tubes of dextrose beef infusion broth were inoculated with 0.2 cc. of the most recent semi-solid cultures (1) of the standard strains of pneumococcus, types I (Neufeld), II and III and incubated at 37°C. for twelve hours to be used as seed cultures; the semi-solid cultures having been plated, fished, agglutinated and tested for purity. After incubation, flasks of broth (300 cc. per flask) were inoculated with 1 cc. of the seed culture, which had been examined and agglutinated against the three types and tested with bile, and incubated at 37°C. for thirteen hours. After flasks had been examined for purity they were heated at 53°C. for one-half hour. The heated culture was then centrifugalized for one-half hour. The broth was poured off and a small amount of 0.85 per cent saline was added to the sediment. This suspension was transferred to a sterile bottle, to which a definite amount of saline was then added and the mixture thoroughly shaken. One cubic centimeter was removed for bacterial count and the remaining suspension was again heated to 55°C. for one-half hour. The vaccine was standardized by the Helber counting-chamber and 0.3 per cent tricresol in a 2 per cent solution was added. Cultural and animal tests were made for sterility and the vaccine was diluted to the desired strength.

The results of the experiments to determine the protective value of a single dose and a series of doses of the vaccine in mice are listed in the preceding tables. The vaccine in these tests was given subcutaneously; the test inoculation of virulent culture, intraperitoneally.

For purposes of comparison, tests of the protective value of pneumococcus vaccination were started in rabbits but discontinued when it was found that the protection, which is obtained in these animals, did not differ essentially from that which was obtained in mice, the protection being limited to the infection with the homologous organism. The results of these tests are recorded in the following tables:

Experiment VII (8-26-19). Rabbits receiving six intravenous inoculations of 1 cc. at six-day intervals of type I vaccine containing six billion organisms per cubic centimeter; tested eight weeks after last inoculation with virulent broth cultures of types I and II

CULTURE—PNEUMOCOCCUS I					CULTURE—PNEUMOCOCCUS II				
Quantity	Number of rabbits inoculated	Number of rabbits lived	Number of rabbits died	Time animal died	Quantity	Number of rabbits inoculated	Number of rabbits lived	Number of rabbits died	Time animal died
cc. 0.1	2	2	0	4 mos.	*cc.* 0.1	2	1	1	72 hrs.
0.1	Control—Normal rabbit, died			41 hrs.	0.1	Control—Normal rabbit, died			70 hrs.

Experiment VIII (8-26-19). Rabbits receiving twelve intravenous (9, 1 cc.—3, 2 cc.) inoculations at three-day intervals of type I broth culture heated to 55°C. for one-half hour and tested eight weeks after last inoculation with virulent broth cultures of types I and II

0.1	2	2	0	4 mos.	0.1	2	0	2	22-70 hrs.

Control as above

In these experiments, rabbits were selected weighing between 1400 and 1800 gms. The virulent cultures were given intravenously and the animals were autopsied and pneumococci recovered from all that died.

CONCLUSIONS

After one dose of three billion pneumococci, type I, there was only very slight protection against type I and none against either types II or III. After three doses of type II vaccine there was slight protection against type II and none against types I and III. After six doses of type III vaccine there was no protection against types I, II, or III. After three doses of type I vaccine there was no protection against either type II or III but definite protection against 0.0001 cc. or 100 times the standard fatal dose of Type I which the control unvaccinated mouse received. Finally, vaccination with six weekly doses of six billion cocci, type I, or a total of thirty-six billion cocci, protected against a virulent inoculation of 0.001 cc. but not against 0.01 cc. of the virulent type I culture.

Definite protection was then obtained against the development of the homologous types of infection when large doses of vaccine were used; but the degree of protection that was obtained was not great considering the quantities of vaccine that were used to vaccinate the animals. The parasitism of such highly virulent cultures is so great that the pneumococci develop in the vaccinated animal just as they grow in a test tube of immune serum. The virulence of pneumococci has been found in previous studies to be largely dependent upon its growth energy or vegetative energy which in the animal tissues constitutes parasitism (2).

REFERENCES

(1) Wadsworth, A. B.: Proc. N. Y. Path. Soc., 1903, 3, 113.
(2) Wadsworth, A. B.; and Kirkbride, M. B.: Jour. Exp. Med., 1918, 28, 791.

SEROLOGICAL RELATIONSHIPS OF LIVER AND KIDNEY

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The subject of immune bodies against various tissues has occupied considerable space in the literature of immunity and especially has emphasis been laid in the discussions on the question of the "specificity" of the antibodies produced against organs. It is particularly with this latter phase of the subject that our experiments have dealt; the results we wish to report here concern however only liver and kidney. We do not desire to go into the literature (1) on this subject at the present time and will only briefly review some of the work which has appeared bearing most directly upon the antibodies produced against renal or hepatic tissue.

Bierry (2) and his co-workers, using so-called (3) nucleo-proteins of various organs (liver and kidney) for immunization, state that following injection of these antisera into animals of the same species as those whose organs had been used for immunization, definite and more marked changes occurred in the organs corresponding to the antiserum injected. They believed therefore that a more or less distinctly specific cytotoxic serum had been developed. Beebe (4) using essentially the same technic reported even more definite results regarding cytotoxins. Armand-Delille and Leenhardt (5) agreed in the main with Bierry but qualified their statements by admitting changes in organs other than those corresponding with the specific sera. Pearce (6) and Pearce, Karsner and Eisenbrey (7), on the other hand, failed to find a distinct selective action of the antisera on the homologous organ. It is evident that the cytotoxic method of determining *in vivo* differentiations between various tissue antisera has not given sharp or clean cut results in all cases and the reports of various investigators are not conclusive in view of the contradictory statements.

Two facts, probably of importance, seem to have been brought out however by various of the above mentioned investigators. Pearce and his co-workers state that the changes which are produced in the organs as a result of the injection of the cytotoxic sera are in large part due to the agglutinins for the erythrocytes which are present in the various anti-tissue sera, and Armand-Delille and Leenhardt arrived at the conclusion that the antisera are composed of a complex of antibodies. The antibodies prepared against the cells of a single organ are therefore apparently complex; really a mixture of several antibodies; it ought to be possible to separate these by absorption of the immune sera and to determine what is left after this absorption by complement fixation tests.

Experiments have previously been carried out in which absorption has been used to determine the relationship of various organs, but on the whole they have been inconclusive because they have not been pushed far enough. Forssner (8) carried out experiments in which he attempted to differentiate serologically various organs by absorption and precipitins but, while he could apparently show certain relationship, he was not able to demonstrate marked differentiation. Michaelis and Fleischmann (9) were able by absorption and complement fixation to show differences between anti-red-blood cell sera and anti-tissue sera in spite of the fact that the serum prepared against the one or the other cell contained antibodies reacting with the non-homologous cells. Rados (10) was not able to determine differences between various organs by the use of antisera in complement fixation tests alone. More recently Kahn and McNeil (11) failed to find complement fixation with antisera prepared against a few tissues. Our experiments have been directed towards determining what differences could be shown between liver and kidney antisera by complement fixation reactions with various antigens before and after absorbing the various sera with cell suspensions of the various organs.

TECHNIC

We used throughout guinea-pig organs for immunization, and rabbits for the production of the antisera. The rabbits were injected four times with an interval of one day between each injection, and were bled on the ninth to eleventh day after the last injection. In most cases the animals were injected intravenously with a suspension of the organ ground in salt solution, and then filtered through sterile gauze. None of the animals so injected showed any immediate ill effects as a result of the injection, but many of them showed a tendency toward loss of weight. A few animals were injected intraperitoneally, but we found that we lost more animals injected in this manner than when injected intravenously. The animals injected intravenously gave sera which were on the whole very slightly more active, but the difference was not sufficiently marked to permit a statement that the intravenous method was better. Practically either method of injection gave satisfactory results. The organs used in immunizing the rabbits were removed from the guinea-pigs at once after killing them; they were not washed but were immediately cut up and ground in a porcelain mortar without attempting to get rid of the serum or of the red blood cells. These two factors were ignored upon *a priori* considerations and will be discussed later. The organ particles were ground without addition of any solution; after they had been reduced to a rather homogeneous semi-fluid mass, a little 0.85 per cent sodium chloride was added and the grinding continued until a smooth paste was obtained. Twenty cubic centimeters of the salt solution were added to each two kidneys or the approximately equal volume of liver tissue and from two to three cubic centimeters were injected into each animal.

The antigens used in the complement fixation tests were prepared in a number of different manners. At first we used organs which had been perfused to remove the red blood cells and serum, and which had become definitely blanched. These were ground in the same manner as were the organs used for injection and the cell suspension repeatedly washed in salt solution by centrifugation; this gave a very satisfactory antigen with kidney but not with liver. The washed kidney cell residue from two kidneys was diluted with ten cubic centimeters of salt solution, and to this 1 cc. of 5 per cent carbolic acid was added as preservative. This served as stock antigen. Antigens prepared with and without carbolic acid were tested and gave identical results. Antigens were prepared in a similar manner excepting that the organs were not washed by perfusion. In a number of cases immediately following the grinding the suspension was filtered through gauze to remove any larger particles and connective tissue which had not been broken up by the grinding. In some antigens we attempted to get rid of the red blood cells by first washing the organ paste with distilled water until the supernatant fluid was no longer discolored, and finally making up the cell suspension with salt solution as above; we discarded this method as the antigens were often not satisfactory. We also used antigens which had been dried rapidly *in vacuo* after being ground and washed as above. By all of these methods we were able to obtain satisfactory kidney antigens.

In the greater part of our work and constantly in the later part, we have used not the organ cells, but the organ extract as antigen. We took the unwashed organs, ground them as above and added about 20 cc. of salt solution, centrifuged for twelve minutes at full speed—approximately 3000 revolutions per minute—and used for the antigen the supernatant fluid which was slightly cloudy or opalescent. By this method we obtained excellent antigens with kidney and it was the only method by which we were able to obtain a satisfactory liver antigen. In preparing the liver antigen we used a quantity of liver tissue approximately equal to the mass of the two kidneys. Occasionally antigens were not satisfactory because they were either anticomplementary, or not antigenic; such antigens were not used. This latter method of preparing the antigen had this advantage—that the erythrocytes were not contained therein; the serum was of course retained but as will be seen this was a factor of little or no importance.

In absorbing the serum with the various organs we used throughout the solid residue of the organs as obtained by grinding and repeated washings (at least four washings were done before the cell suspension was used as absorbent). We extracted both diluted and undiluted sera, but found it more satisfactory to work with the undiluted serum. The serum and organ were left in contact for thirty minutes at 37°C. and then were separated by centrifuging. In some cases the extraction was repeated. We used, as a rule, a volume of tissue cells equivalent to one-fourth the volume of the serum.

At times, and especially after the sera had been in contact with either kidney or liver, they proved to be anticomplementary after the absorption. This fact caused us considerable difficulty for some time but we finally found a method for removing this anticomplementary activity. After the serum had been separated from the organ mass we added a considerable quantity of barium sulphate, about one-fourth the volume of the serum, shook the mixture and then centrifuged, separated the serum and then inactivated. It seems probable that the barium sulphate carried down fine particles of the absorbent which had remained suspended in the serum and that the inactivation played a negligible part in the restoration of the serum.[1]

The technic of the complement fixation reactions as carried out in these experiments was relatively simple. As hemolytic system, we used throughout sheep corpuscles, rabbit anti-sheep red blood cell serum, and guinea-pig complement. The latter was used in three unit quantities. The corpuscle suspension used was always a very dilute one, approximately a 0.5 per cent suspension, as we desired our end results to be either clearly positive or negative. Every day before carrying out the actual series of reactions we had in mind, we tested the anticomplementary and antigenic dose of the antigen; the antigen was then used in a quantity well under the anticomplementary dose (less than half of the anticomplementary dose) and in a quantity representing three to four units of the antigen. Actually we usually used the kidney antigen in a dose of 0.1 to 0.3 cc. of a one to ten dilution of the stock cell suspension or in 0.1 to 0.05 cc. of a one to five dilution of the extract antigen; the liver antigen was usually used in quantities between 0.3 to 0.1 cc. of a one to five dilution of the extract.

Before we turn to the consideration of the results obtained, certain preliminary considerations may well be taken up which have an influence upon the interpretation of the more specific results dealing with the relationships of kidney and liver antisera.

We examined the serum of a number of animals to determine whether there exist normally in the blood of rabbits, antibodies giving fixation with liver or kidney cells or extracts of such cells of the guinea-pig. The sera of ten normal rabbits were examined; only one serum gave fixation with kidney antigen. It is doubtful whether this represents a true fixation as this serum which gave fixation was freshly drawn and had been inactivated; it seems probable that we were dealing with a nonspecific fixation such as described by Kolmer and Trist (12) as being present after inactivation of rabbit serum. It appears, therefore, that normal rabbit serum contains no antibodies reacting in the complement fixation reaction with guinea-pig cells. This fact, however, in view of the one exception mentioned will have to be further investigated.

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[1] This method of removing anticomplementary substances (?) from serum has been tried out with a small number of anticomplementary sera sent in for the Wassermann reaction and seems to have some practical value but as yet sufficient data has not been collected to warrant any definite conclusion as to either the usefulness or limitations of this method.

We have carried out several experiments to determine whether the immune sera would show complement fixation when inactivated guinea-pig serum was used as antigen. Using several different antisera, both anti-kidney and anti-liver, and using the guinea-pig serum in quantities of either 0.1 or 0.2 cc., we did not obtain positive fixation even when 0.1 cc. of the immune sera were used. It is evident therefore that the serum content of the extract antigen used in our later experiments (which dose of course contained the serum content of the organs) cannot be an interfering factor in the reactions to be described. Furthermore, the quantity of serum in the diluted antigen used in the complement fixation reaction was even less than the largest quantities which did not give fixation.

In the case of the guinea-pig erythrocytes the conditions are not so simple. We examined the anti-organ sera for agglutinins, hemolysis and complement fixing antibodies against guinea-pig corpuscles and found that such antibodies were present in both anti-liver and anti-kidney serum; using 0.25 cc. of a 5 per cent suspension of guinea-pig corpuscles we found that with anti-kidney serum 0.005 cc. produced agglutination, while with the anti-liver serum 0.001 cc. gave agglutination. The hemolysins were, however, not so active as were the agglutinins, as 0.05 cc. of both sera gave only partial hemolysis of 0.25 cc. of a 5 per cent suspension of guinea-pig corpuscles. It was evident from these results that the presence of corpuscles in the antigen might well be a factor in the complement fixation reaction. In table 1 we give the results of complement fixation reactions in which various suspensions of guinea-pig corpuscles were used as antigen. We do not give here—and will not give in later tables—the details of the reactions as these points have been covered in the description of the technic.

It will be noted that the anti-liver serum contained a greater concentration of complement fixing antibodies reacting with guinea-pig corpuscles than did the anti-kidney serum; this fact had also been noted in connection with the agglutinins. As to the possibility of this presence of anti-erythrocyte antibodies influencing the reactions which we report here, we believe that in the antigen used in our experiments, that is, in the quantity used in our work, red blood cells were not present in a quantity corresponding to 0.5 cc. of a 0.1 per cent suspension; in this quantity and even in the lower quantity, which did not give fixation with either serum, the mixture of serum, complement and cell suspension showed a definite reddish tinge and was distinctly clouded by the corpuscles; on the other hand the antigens did not show this tinge in the dilutions and quantities used. Furthermore, the volume of the ground organ rarely exceeded 2 cc.; in order to have a 0.1 per cent suspension of erythrocytes in the final dilution of the antigen one-twentieth of this bulk would have been composed of red blood cells which certainly was not the case. Therefore, in those cases in which the cells of the organs were used as antigen, it is very unlikely that antibodies

TABLE 1

Complement fixation of anti-liver and anti-kidney serum with varying suspensions of guinea-pig corpuscles

QUANTITIES OF GUINEA-PIG CORPUSCLE SUSPENSION	1 PER CENT SUSPENSION		0.1 PER CENT SUSPENSION	
	0.5 cc.	0.25 cc.	0.5 cc.	0.25 cc.
Anti-kidney serum				
cc.				
0.1	++++	0	0	0
0.07	0	0	0	0
Anti-liver serum				
0.1	++++	++++	++++	0
0.07	++++	++++	++++	0

reacting with red blood cells played any part in the reaction. Of course in those later experiments in which we used the organ extracts the corpuscles could evidently have had no influence.

CROSS FIXATION WITH KIDNEY AND LIVER ANTIGEN

Considering first the relation of the antisera to the two antigens, we found that in all experiments the anti-kidney serum gave complete fixation with its homologous antigen in smaller quantities than when tested against liver antigen; in four cases out of six the anti-liver serum also fixed in smaller quantities with its homologous antigen than it did with kidney antigen, in the two other cases with liver antiserum, fixation was obtained with identical quantities of the serum with both antigens—in both of these latter cases we were probably dealing with rather weak liver antigens as shown by the poor fixation with anti-kidney serum. When we compared two different antisera in fixation reactions with a single antigen, we found a tendency towards fixation in smaller quantities when antiserum and corresponding antigen were used. This relation did not appear constantly for in some cases the anti-liver serum showed with kidney antigen stronger fixing powers than did the anti-kidney serum. This fact will be referred to again later.

TABLE 2

Cross fixation of sera and antigens

	CUBIC CENTIMETERS OF SERUM					
	0.03	0.01	0.008	0.006	0.004	0.002
Kidney antigen						
Anti-kidney serum	++++	++++	++++	++	+	0
Anti-liver serum	++++	+++	+++	+	0	0
Liver antigen						
Anti-kidney serum	++++	0	0	0	0	0
Anti-liver serum	++++	++++	++++	++++	++	0

From the above results it is evident that there exists a definite even though not absolutely sharply defined tendency toward a differentiation between these two organs as shown by cross fixation.

ABSORPTION EXPERIMENTS WITH LIVER AND KIDNEY

We will consider first only the relationship existing between similar sera and antigens absorbed with different organs.

TABLE 3

	CUBIC CENTIMETERS OF SERUM								
	0.05	0.04	0.03	0.02	0.01	0.008	0.006	0.004	Control
a. Kidney antigen and anti-kidney serum									
Unabsorbed	++++	++++	++++	++++	++++	+	0	0	0
Absorbed with kidney	++++	++	+	0	0	0	0	0	0
Absorbed with liver	++++	++++	++++	++	0	0	0	0	0
b. Liver antigen and anti-liver serum									
Unabsorbed	++++	++++	++++	++++	++++	++++	++++	+++	0
Absorbed with kidney	++++	++++	++++	++++	++++	0	0	0	0
Absorbed with liver	0	0	0	0	0	0	0	0	0

From table 3, which is characteristic of the results obtained in a considerable number of experiments, it is evident when we had the combination of absorption of an immune serum by the organ against which it had been prepared, and when we tested the complement fixing power with a homologous antigen, that we found definitely more of the antibodies had been removed than when a non-homologous organ had been used for the absorption. This statement applies equally well in the case of liver as it does in the case of kidney. We find that a certain amount of antibodies was removed by the non-homologous tissue but that from three to five times as much was removed by the homologous tissue. There was also evident a tendency for liver tissue to absorb relatively more than kidney tissue. The relationship as revealed when we tested the various sera against the non-homologous antigens is shown in table 4.

We found here when we tested the fixation of the antisera after absorption with an antigen which was not homologous with the serum, but which was homologous with one of the absorbents, that in the case of the anti-liver serum the evidence of the definite relationship of the antiserum to the organ against which it had been prepared and by which it had been extracted, still was evident; but that in the case of the anti-kidney serum this relationship did not appear, and quite as much of the antibodies fixing with liver antigen had been removed from the anti-kidney serum by liver as by kidney. However, when we carried out simultaneous experiments in which we tested the various absorbed antisera of liver or kidney against the two different antigens, we found that relatively less of the antibody content had been removed by the non-homologous absorbent when tested with the antigen corresponding to the serum, than when tested with the non-corresponding antigen, that is, in this latter case the antigen corresponding to the absorbent. We found, therefore, that there exists not only a relationship between the anti-

TABLE 4

	CUBIC CENTIMETERS OF SERUM									
	0.05	0.04	0.03	0.02	0.01	0.008	0.006	0.004	0.002	Control
a. Liver antigen and anti-kidney serum										
Unabsorbed	++++	++++	++++	++++	0	0	0	0	0	0
Absorbed with kidney	++++	++++	++	0	0	0	0	0	0	0
Absorbed with liver	+++	0	0	0	0	0	0	0	0	0
b. Kidney antigen and anti-liver serum										
Unabsorbed	++++	++++	++++	++++	+	+	0	0	0	0
Absorbed with kidney	+++	+++	+++	+	0	0	0	0	0	0
Absorbed with liver	++	0	0	0	0	0	0	0	0	0

serum and its corresponding antigen which could be shown by absorption but also a relationship between absorbent and corresponding antigen when tested with non-corresponding serum. It therefore becomes evident that the relationship existing between antisera against various organs is not simple but that probably a considerable number of factors must be taken into consideration.

ABSORPTION EXPERIMENTS WITH OTHER ORGANS

We next tested the fixation of the two antisera against their homologous antigens after absorption with organs other than liver or kidney. We first tried absorption with washed guinea-pig red blood cells, and found that by this means relatively small quantities of the antibodies were removed. Spleen was also tested and removed very little, and finally brain was used as absorbent and was found to remove possibly a little more than either red blood cells or spleen. Probably not as much was absorbed from the kidney serum by brain as by liver; very nearly as much was absorbed from the anti-liver serum by brain as by kidney; there was however a tendency for the kidney to absorb slightly more than the brain in some experiments, and in no case did the brain absorb more from the liver serum than did the kidney.

We carried out a series of experiments in order to determine whether the differences in absorption might be influenced by variations in the volume of the absorbent material. When we used similar volumes of the absorbent we noted that the absorbent corresponding to the antiserum showed, as stated, distinctly greater action on the serum and even when we used twice as much of the non-homologous absorbent the homologous absorbent still had removed more reacting substances. In the case of the comparison of the non-homologous absorbents, however, the quantities of the absorbent did apparently play a part, and if the quantities of the two non-homologous absorbents were not nearly the same the above mentioned relationships were confused. This fact had no influence on the results given above as they were carried out with similar quantities of the various organs used for absorption. This marked influence of the variations of the quantities of the non-homologous absorbents suggests that there was here a purely physical absorption occurring in which chemical relationship played little or no part and we tested two inert absorbent substances not of animal origin, namely, kaolin and barium sulphate, and found that neither of these two substances removed any of the antibodies. While, therefore, as a result of these quantitative experiments, the influence of the use of non-homologous tissues as absorbents and their inter-relation is not yet clear, nevertheless the definite relationship between homologous absorbent and anti-serum is clearly demonstrated.

FIXATION WITH ANTIGENS OTHER THAN LIVER OR KIDNEY

We tested the anti-kidney and anti-liver sera against two other organ antigens, namely, spleen and brain, and found that both antisera gave fixation with these antigens. In general anti-liver serum gave better fixation than did anti-kidney serum with these non-homologous antigens. When we tested the various absorbed sera against these two antigens we obtained results which were in the main confirmatory of those reported above; however, there was a less marked regularity of results in these experiments than in those above. On the whole we can say that the definite relationship between the antiserum and the homologous absorbent was usually evident, in that these sera (serum absorbed with its homologous tissue) showed less fixing power than the other absorbed sera. However, in a few cases the relationship between the absorbent and the corresponding antigen became more evident than the relationship between antiserum and absorbent; in such cases the immune serum absorbed with the tissues corresponding to the antigen gave least fixation. Usually there was very little difference between the results on the one hand with serum and corresponding absorbent, and on the other with absorbent and corresponding antigen, when the same serum and antigen was used and only the absorbent varied. In practically all cases we could note evidence of a definite relationship between the absorbent and corresponding antigen, which showed itself in the fact that the serum which had been absorbed with the tissue corresponding to the antigen, showed less fixation than did the same serum with the same antigen after being absorbed with other tissues—excepting of course the tissue corresponding to the anti-serum. One exception to this rule did occur at times, and this was in the case of anti-kidney serum absorbed with liver tissue; here liver tissue apparently possessed a very strong absorbent power. We cannot at the present time offer an explanation for this result but it seems to correspond with the more active part the liver seems to take throughout these experiments in the production of antibodies.

DISCUSSION

Summarizing now the facts that have been brought out in these experiments we found that anti-liver and anti-kidney sera gave positive results in complement fixation reactions, not only with the corresponding antigens, but also with antigens prepared from other organs of the same species; however, in cross fixation experiments there was a tendency for definite relationship between antiserum and corresponding antigen to become apparent. Furthermore, it appeared that anti-liver serum tended to give, in general, complement fixation in smaller quantities than did the anti-kidney serum. When, however, we tested the complement fixation of the antisera after absorption by various tissues we found marked evidence of a relationship between the antiserum and its homologous absorbent especially when tested with the homologous antigen, which latter relationship was constant and definite. When we tested the various absorbed antisera against non-homologous antigens we still noted a distinct tendency for the appearance of this relationship between antiserum and homologous absorbent, but in some cases this relationship was not so clear, due to the appearance of a relationship between the antigen and corresponding absorbent.

We can put aside the possibility that these reactions are due to the presence of either precipitins or fixing bodies reacting with the guinea-pig serum. That the antibodies reacting with red blood cells play any part is unlikely from what we have noted regarding the quantitative relationships of the antibodies reacting with the guinea-pig red blood cells, and especially since we have found that after absorption by the various tissues the anti-liver and anti-kidney sera no longer contain agglutinins for the guinea-pig red blood cells. We were therefore apparently dealing in these experiments with reactions to the tissue antigens, and the erythrocytes as antigens influenced the results little, if at all.

We can therefore first state that there exist, in the antisera prepared against liver and kidney, antibodies, which have a rather wide range of activity, at least as far as other organs of the guinea-pig are concerned, giving fixation not only with liver and kidney but also with spleen and brain. It seems, therefore, that these two organs contain antibody-producing substances which have common relationships with numerous other organs of the body and possible with all tissues of the guinea-pig. These would correspond with so-called species specific antibodies.

As a result of the complement fixation experiments with absorbed antiserum and the homologous antigens, the definite relationship between antiserum and absorbent is evident. It certainly appears that each organ contains quantities of antigenic and antibody-producing substances which are more characteristic of that organ than of other organs; that the antibody producing effect of the organ is most active in connection with these anti-characteristic substances, and that because of the greater content in the antiserum of bodies reacting with these substances and the greater content of the tissue in these characteristic substances, absorption of antibodies is greater when we mix the corresponding antiserum and absorbent. This relationship of the antisera and organs would correspond to the so-called organ specificity or would signify, at least, a quantitative organ specificity.

When, however, we study further the experiments in which we used non-homologous absorbents and carried out complement fixation reactions with non-homologous antigens, we find a tendency for a definite relationship to appear between the non-homologous absorbent and its corresponding antigen. This tendency is fairly regularly apparent and it seems that we can interpret it best by assuming that in each organ there exist certain substances which have a relationship to definite organs other than the one actually used as the immunizing agent. It is hardly possible that this relationship is identical with the first relationship which we have spoken of above, that is, the relationship which apparently is common to all organs of a species, in view of the very definite limitation of the relationship which appears in these experiments. However, at the present time, we offer this only as a possible explanation as we feel that this work must be extended to include a consideration of the interrelation of a number of other organs.

It is however evident, as would be expected, that the immune sera prepared against tissues such as liver and kidney are complex in their nature, that they are composed of a number of different antibodies, probably varying in their relationship, some rather limited in their range of action and others with a wider range of action. It would seem that the liver contains more of these last type than does the kidney. Three facts seem to bear out this conclusion regarding the liver: We find a tendency for liver to absorb relatively more antibody from anti-kidney serum than does kidney from anti-liver serum; the liver serum reacts better with the non-homologous antigens than does anti-kidney serum; and finally the liver used as an absorbent in the experiments with non-homologous antigen tends at least in some cases to approach the absorbing action of the tissue corresponding to the antigen used.

This suggests the question of the relationship of various organs to each other; a matter which has been considered by several previous investigators (Forssner (8), Cesaris Dehmel and Scotti (13), Fiessinger (14) and Fleischmann and Davidsohn (15)). We do not think it advisable to go into this matter at the present time, but desire to push our work further considering various other organs before we venture upon a discussion of this matter.

We have throughout avoided the use of the term "organ specific," and have done so intentionally, There has come to be considerable confusion regarding the use of the term specificity in connection with serological reactions, and we do not care at this time to enter into a discussion of the use of this word. We are satisfied with the statement that we have been able to demonstrate a definite relationship between anti-liver serum and its homologous antigen and between anti-kidney serum and its homologous antigen. Whether similar definite relationships can be shown with other organs is now under investigation.

CONCLUSIONS

By means of complement fixation reactions and absorption of sera prepared against guinea-pig liver and kidney we have been able to show that there exists a definite relationship between the anti-organ sera and the homologous antigens.

The antigens and antisera are not simple but are complex in nature and probably are composed of several different partial antigens and immune bodies.

Possibly these partial antigens and antibodies can be arranged in three groups: The first having a very wide range of activity and having a relationship to all or practically all tissues of the species; the second having a limited range of activity and having relationship only with the tissue used in the preparation of the antiserum; and the third being possibly a group of antibodies, also rather limited in their range of activity but reacting only or more strongly with individual tissues other than the one used as the immunizing substance.

REFERENCES

(1) References to the literature of cytotoxins will be found in the following articles: Sachs: Biochem. Cent., 1903, **1**, 573; Rössle: Lubarsch-Ostertag. Ergb. d. Path., 1909, **13-2**, 124; Sobernheim, Krehl and Marchand: Handb. d. Allg. Path., 1908, **1**, 535; Landsteiner: Oppenheim's Handb. d. Bioch., 1910, **2**, pt. 1, 542.

(2) Bierry: Compt. Rend. Soc. Biol., 1903, **55**, 476; Bierry and Pettit: idem., 1904, **56**, 238; Bierry and Mayer: idem., 1904, **56**, 1016; Bierry, Pettit and Schaeffer: idem., 1907, **63**, 496 and 566.

(3) **Wells**: Zeitsch. f. Imm., Orig., 1913, **19**, 599.
(4) **Beebe**: Journ. Exper. Med., 1905, **7**, 733; Brit. Medical Journ., 1906, **2**, 1786; Science, 1908, **27**, 648.
(5) **Armand-Delille and Leenhardt**: Compt. Rend. Soc. Biol., 1907, **62**, 31.
(6) **Pearce and Jackson**: Journ. Inf. Dis., 1906, **3**, 742; **Pearce and Sawyer**: Journ. Med. Res., 1908, **19**, 269.
(7) **Pearce, Karsner and Eisenbrey**: Journ. Exper. Med., 1911, **14**, 44.
(8) **Forssner**: Muench. med. Woch., 1905, **52**, 892.
(9) **Michaelis and Fleischmann**: Zeit. f. Klin. Med., 1906, **58**, 463.
(10) **Rados**: Zeit. f. Imm., 1913, **19**, 579.
(11) **Kahn and McNeil**: Journ. Imm., 1918, **3**, 277.
(12) **Kolmer and Trist**: Journ. Inf. Dis., 1916, **18**, 64.
(13) **Cesaris-Dehmel and Scotti**: Arch. p. le Scienze Mediche, 1907, **31**, 135.
(14) **Fiessinger**: Compt. Rend. Soc. Biol., 1907, **62**, 671; idem., 1907, **63**, 573.
(15) **Fleischmann and Davidson**: Fol. Seriolog., 1908, **1**, 173.

ON THE PLACENTAL TRANSMISSION OF SO-CALLED NORMAL ANTIBODIES

III. ANTILYSINS

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In completion of the present series of studies on the placental transmission of so-called normal antibodies the following observations as regards antilysins are reported.

A. ANTI-MEGATHERIOLYSIN

The examination for the presence of these bodies in the blood of the mother animal and the kid, as well as in the milk, was carried out in accordance with the quantitative method employed by the Serum-Institute for measuring antihemolytic bodies. It is to be observed that in the examination of milk only approximate accuracy can be obtained owing to its opacity With the purpose of overcoming this obstacle the casein in one experiment was precipitated with rennet, but this procedure was abandoned as it was found that the antihemolytic bodies were also almost entirely carried away.

The samples were inactivated for one-half an hour at 56°C., after it had been proved by experiments that the megatheriolysin-neutralizing power was practically unweakened by this process. The results of the entire series of experiments are given in the appended table, whereas only a single series is diagrammatically represented in the following curve (chart 1).

It appears that the titer of the blood of the mother animal follows an average constant; this also applies to the blood of kids, where the titer in all cases was found to be lower than in that of the mother animal, and in the case of twins it was very nearly the same. The directly determined figures remained very nearly constant during the period of the research, whereas the weight-correlated figures exhibited a fairly gradual rise.

The titer determinations for the milk were very low.

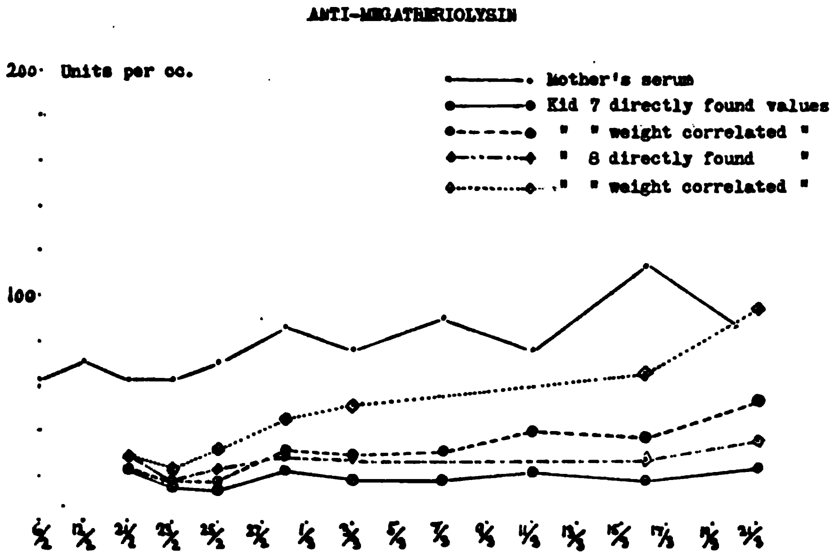

CHART 1

B. ANTI-VIBRIOLYSIN

In a work, of which unfortunately only a brief summary has been available, Schenk (1) maintains that the anti-vibriolysin content is the same in the blood of the mother as in that of the child; for which reason he concludes that these bodies are easily diffusible. This finding is corroborated in the case of goats and kids by the researches detailed below. The researches in all comprised five kids and their mothers. In testing for antilysin in the case of two of the kids (11 and 22) and the corresponding mother animal, horse blood as well as goat blood was used as an indicator; in the case of the others only horse blood was employed. The measuring technic and the method of inactivation was the same as in the tests for megatherio-lysin.

The results, as appearing from the table, are that the titer of the blood is very low and nearly the same in mother and kid; further, that in the course of the period of the research it fluctuates somewhat, but without any particularly marked tendency in either direction except that the weight-correlated titers show in some cases a definite rise.

In the two cases in which samples of milk were investigated, the amount of antilysin was rather great at the parturition, then it decreased rapidly but later on it increased again in some degree.

C. ANTI-STAPHYLOLYSIN

As in the case of the normal anti-vibriolysin, Schenk found that the uterus blood and the funicular blood contained the same quantities of anti-staphylolysin; and further that these normal antibodies are found in woman's milk as well as in goat's and cow's milk. Polano, however, found that, although the blood of both mother and child contained anti-staphylolysin, it was found in the former in larger quantities than the latter.

The examination of the normal anti-staphylolysin was undertaken in the same manner as for the above mentioned antihemolysins, namely, goat blood was used as indicator and the samples were inactivated for one-half hour at 56°C. These examinations of the serum samples showed that the maternal sera at the time of parturition contained larger quantities of normal anti-staphylolysin than did the sera of the kids; in this respect the serum of kid 31 forms no definite exception in view of the fact that the first blood sample was taken twelve hours after birth. The titer of the maternal sera fluctuated rather strongly, but with no definite tendency in either direction.

As regards the titer for the kids, it increased in most cases during the first days after birth, but soon afterwards it decreased, after which it remained more or less constant, although in several cases one and a half months after birth it showed a secondary increase; this latter increase possibly bears some relation to the increase in titer of the milk, which frequently occurred at a somewhat earlier period. This relationship, however, could not be established with certainty, partly because the kids at that period take other nourishment, and partly because they do not always confine themselves to suckling their own mother. This antilytic action of the milk can be reduced by skimming it and so may partially proceed from the cream.

These experiments indicate that the normal antistaphylolysin is not always, as is claimed by Schenk, transmitted quantitatively to the young. My results, accordingly agree with those of Polano (2).

D. ANTI-SAPONIN

As is well known, it was demonstrated by Ranson (3) in 1901 that the constituent in the serum which neutralizes saponin and hence inhibits its hemolytic action is cholesterin; and further it was proved by Madsen and Noguchi (4) in 1904 that this binding admits of a quantitative measurement. Accordingly, as in this process "antigen" as well as "antibody" are known, it is of interest through the medium of saponin binding to examine the occurrence of cholesterin in the sera of goats and their new-born kids, as well as its presence in the maternal milk. As far as I know, such examinations have not been undertaken for the life-period dealt with in the present article. For a description of the methods used in titrating the samples reference is made to the process described by Madsen and Noguchi.

It appears from the appended table (I, d) that the titer of the mother animal remains nearly constant, before as well as after parturition, whereas that of the young increases directly after birth until it reaches a higher point than that of the mother, and then, as a rule, rapidly decreases; only the titer of the kid 15 and to a certain extent kid 13 (1914) remained nearly constant; later in one or two cases an increase was again observed.

If one looks at the weight correlated titers, it will appear that after the primary increase, which, as a rule, is followed by a decrease, the titer on an average increases gradually, so that the total content in the blood of the saponin-neutralizing bodies is upon the whole increasing. Only in kid 15, however, is the increase nearly an uninterrupted and gradual one.

TABLE 1

Titration of antilysins in material and kids' sera

Dates of taking serum samples

* Birthdays. a, Titer found directly; b, weight factor†; c, titer correlated to weight

A. Anti-megatheriolysin

NUMBER OF KID	MOTHERS' SERUM												
4	12/2	*14/2	16/2	21/2	25/2	28/2	4/3	9/3	11/3				
	172	134	172	178	209	172	191	162	172				
5 x 6 (1914)	12/2	*21/2	23/2	25/2	28/2	3/3	7/3	11/3	16/3				
	139	100	105	120	110	137	138	138	138				
7 x 8	6/2	12/2	*21/2	23/2	25/2	28/2	3/3	7/3	11/3	16/3	20/3		
	63	71	63	63	71	87	77	91	77	114	87		
9 x 10	6/2	12/2	*24/2	26/2	28/2	3/3	7/3	11/3	14/3	17/3	21/3	25/3	30/3
	94	100	100	88	100	108	115	115	117	87	117	94	94
6 (1915)	*22/2	27/2	2/3	6/3	10/3	15/3	22/3						
	33	54	54	54	67	67	44						

NUMBER OF KID	KIDS' SERUM											
4		*14/2	16/2	18/2	21/2	25/2	28/2	4/3	9/3	11/3		
	a	44	40	63	72	59	49	38	45	38		
	b		1.1		1.3	1.6	1.8	2.0	2.3	2.4		
	c		44		100	94	88	76	112	91		
5 x 6 (1914)		*21/2	23/2	25/2	28/2	3/3	7/3	16/3				
	6a		30	29	33	29	22	22				
	b		1.1	1.2	1.4	1.6	1.8	2.4				
	c		33	35	46	46	40	53				
	5a	50										
7 x 8		*21/2	23/2	25/2	28/2	3/3	7/3	11/3	16/3	21/3		
	7a	23	15	14	23	19	19	23	19	25		
	b		1.1	1.3	1.4	1.6	1.7	1.8	2.0	2.2		
	c		17	18	32	30	32	41	38	55		
	8a	29	18	23	29	29			29	37		
	b		1.3	1.4	1.6	1.8			2.3	2.6		
	c		23	32	46	52			67	96		
9 x 10		*24/2	26/2	28/2	3/3	7/3	11/3	14/3	17/3	21/3	25/3	30/3
	9a	28	19	19	26		23		31	31	31	31
	b		1.2	1.4	1.6		2.2		2.6	2.9	3.2	3.5
	c		23	27	42		50		81	90	99	109
	10a	23	23	20	22	20	32	28	35	28	37	29
	b		1.1	1.3	1.5	1.6	1.8	2.0	2.1	2.3	2.5	2.8
	c		25	26	30	32	58	56	74	64	93	81
6 (1915)		*22/2	23/2	27/2	2/3	6/3	10/3					
	6a		10	17	14	13	12					
	b		1.1	1.2	1.3	1.5	1.7					
	c		11	20	18	20	20					

NUMBER OF KID	MILK								
4	*14/2	16/2	21/2	9/3	11/3				
		<0.5	0	0	0				
5 x 6 (1914)	*21/2	23/2	28/2	7/3	16/3				
	0	0.7	0	0	0.7				
7 x 8	*21/2	23/2	25/2	28/2	3/3	7/3	11/3	16/3	21/3
	<2	1.2	0	0	<2	0	0	0	0
9 x 10	*24/2	26/3	3/3	7/3	14/3	17/3	25/3		
	1.3	0	0	0.5	0	0	0		
6 (1915)	*22/2	23/2	27/2	2/3	6/3	10/3	19/3	22/3	
	2.0	2.0	1.0	1.0	0.5	0.5	0.5	<0.5	

† These figures represent the ratio between the weight at birth and the weight at the taking of samples. The quantitative results signify antilysin units per cubic centimeter.

TABLE 1—*Continued*

B. Anti-vibriolysin

NUMBER OF KID	MOTHERS' SERUM																		
1	*1/2	6/2	7/2	9/2	11/2	14/2	16/2	18/2											
	5.3	1.3	2.0	2.1	1.3	1.4	1.2	1.3											
3	6/2	*9/2	10/2	12/2	14/2	16/2	19/2	21/2	28/2										
	1.3	1.1	1.0	1.0	0.8	0.8	1.0	0.8	0.8										
11	Indicator: blood of goat																		
	5/2	12/2	*25/2	27/2	2/3	4/3	7/3	11/3	14/3	17/3	21/3	26/3	30/3						
	2.7	4.0	2.0	4.0	2.7	3.0	3.0	3.0	3.0	3.0	3.0	2.7	2.7						
11	Indicator: blood of horse																		
	2.2	3.5	2.2	3.5	2.5	2.5	2.2	2.5	2.2	2.2	2.5	2.1	2.5						
22	Indicator: blood of goat																		
	24/2	*3/3	5/3	7/3	10/3	14/3	17/3	21/3	25/3	28/3	1/4	4/4	8/4	11/4	15/4	21/4	25/4	1/5	15/5
	3.3	3.6	3.9	2.7		2.7	3.4	3.4	3.4	3.4	6.6	3.0	2.8	2.4	2.4	3.0	3.0	2.5	3.3
22	Indicator: blood of horse																		
	2.1	*1.7	2.2	2.2	3.9	2.0	2.0	2.2	2.2	2.1	3.3	1.5	1.4	1.6	1.8	1.7	2.1	1.8	4.0
30	*10/2	12/2	15/2	19/2	23/2	27/2	2/3	6/3	10/3	15/3									
	1.3	1.3	1.3	1.0	1.0	1.0	0.9	0.9	1.6	2.0									

NUMBER OF KID		KIDS' SERUM															
1		*5/2	6/2	7/2	9/2	11/2	14/2	16/2	18/2								
	a	2.0	2.0	1.9	1.9	1.4	1.5	1.0	1.0								
	b						1.3	1.4	1.6								
	c						2.0	1.4	1.6								
3		*9/2	10/2	11/2	12/2	14/2	16/2	19/2	21/2	25/2	28/2						
	a	2.0	2.0	2.2	2.2	2.2	1.5	2.0	1.5	2.0	1.5						
	b		1.0	1.0	1.0	1.06	1.1	1.3	1.3	1.4	1.5						
	c					2.3	1.7	2.6	2.0	2.8	2.3						
11		Indicator: blood of goat															
		*25/2	27/2	2/3	4/3	7/3	11/3	14/3	17/3	21/3	26/3	30/3					
	a	3.0	4.0	1.6	3.0	3.0	3.0	2.5	2.7	3.0	2.7	2.7					
	b		1.2	1.2	1.4	1.5		2.0	2.2	2.4		2.9					
	c		4.8	1.9	4.2	4.5		5.0	5.9	7.2		7.8					
11		Indicator: blood of horse															
	a	1.0	1.3	1.3	1.6	1.6	2.0	1.6	1.1	1.1	1.0	1.1					
	b	As above															
	c		1.6	1.6	2.2	2.4		3.2	2.4	2.6		3.2					
22		Indicator: blood of goat															
		*3/3	5/3	7/3	10/3	14/3	17/3	25/3	28/3	1/4	8/4	11/4	15/4	21/4	25/4	1/5	15/5
	a			3.7	3.7	3.8	3.7	4.2	3.9		3.3	7.7	3.9	3.3		3.9	3.8
	b		1.1	1.2	1.3	1.5	1.6	1.9	2.0	2.3	2.6	2.8	3.0	3.3		3.8	
	c			4.4	4.8	5.0	5.9	8.0	7.8		8.6	21.6	11.7	10.9		14.8	
22		Indicator: blood of horse															
	a	1.5	2.0		3.0	2.4	2.9	2.8	2.5	2.1	1.7	3.4	2.1	1.7	1.7	1.7	1.6
	b	As above															
	c		2.2		3.9	3.6	4.6	5.3	5.0	4.8	4.4	9.5	6.3	5.6		6.5	
30		*10/2	12/2	15/2	19/2	23/2	27/2	2/3	6/3	10/3							
	a	1.0	1.3	1.3	1.3	1.3	0.8	1.0	0.8	1.0							

NUMBER OF KID	MILK										
22	*3/3	5/3	7/3	10/3	25/3	28/3	1/4	11/4	21/4		
	5.0	2.9	2.7	8.0	1.3	<0.5	<0.5	<0.5	<0.5		
30	*10/2	11/2	12/2	13/2	15/2	23/2	27/2	6/3	16/3	19/3	22/3
	8.3	2.7	1.3	0.8	0.5	0	0	1.0	1.5	<1	<1

C. Anti-staphylolysin

NUMBER OF KID	MOTHERS' SERUM												KIDS' SERUM														MILK												
15	*2/3	4/3	7/3	14/3	17/3	21/3	25/3	28/3	1/4	6/4	11/4			*1/3	2/3	4/3	7/3	14/3	17/3	21/3	25/3	28/3	1/4	6/4					25/3	1/4	6/4								
(1914)	25	20	20	25	9.1	20	20	15	15	6.7	15		a	2.0	2.5	6.0	22.7	4.2	6.8	2.7	3.5	6.0	6.7	5.7					67	>50	50								
													b				1.3	1.6	1.8	2.0	2.2	2.3	2.5	2.8															
													c				29.5	6.7	12.2	5.4	7.7	13.8	16.8	16.0															
31	9/3	*10/3	13/3	16/3	20/3	24/3	28/3	1/4	4/4	8/4	11/4	20/4		9/3	*10/3	13/3	16/3	20/3	24/3	28/3	1/4	4/4	8/4	11/4	15/4	20/4		10/3	13/3	26/3	28/3								
(1914)		0.8	2.0	2.0	<1	2.7	1.6	6.5	5.6	6.4	2.7	1.4	a		7.0	6.2	3.4	4.2	3.2	2.2	6.5	2.7	2.7	2.4	2.7	10.0		c.12	c.12	c.12	33								
													b			1.1	1.3	1.5	1.7	1.8	2.1	2.1	2.4	2.6	2.8	3.1													
													c			6.8	4.4	6.3	5.4	4.0	13.7	5.7	6.5	6.24	7.6	31.0													
37x38	*25/3	27/3	1/4	4/4	8/4	28/4	7/5	15/5						*25/3	26/3	1/4	8/4	18/4	23/4	2/5	7/5						*25/3	26/3	28/3	1/4	4/4	8/4	15/4	18/4	23/4	28/4	2/5	7/5	15/5
																											Uns	kim	med	mil	k								
(1914)	10	12	12	6.1	12	8.9	10	8.8					37a		4.5		3.4		1 8								5.5	3.0	8.0	3.0	2.0	4.0	5.5	4.0	22	17	14	11	33
													b		1.1		1.6																						
													c		5.0		5.4																						
																											Ski	mm	ed	mil	k								
													38a	6.1		3.5	3.5	2.8	6.4	6.8	2.0						5.5	1.3	<1	<1	<1	<1	2.7	<1	5.5	0.5			0.8
													b			1.4	1.9	2.1			2.8																		
													c			4.9	6.4	5.9				5.6																	
12x13	*25/2	27/2	2/3	6/3	10/3	13/3	16/3	19/3	22/3	25/3	30/3	3/4	12	*25/2	27/2	2/3	6/3	10/3	13/3	16/3	19/3	22/3	25/3	30/3	3/4		*25/2	2/3	6/3	10/3	13/3	16/3	19/3	22/3	30/3	3/4			
(1915)	1.0	1.0	1.0	4.0	4.0	5.4	1.0	4.0	2.8	4.0	1.0	8.3	a	0	22.5	3.0	1.9	1.9	1.8	1.5	2.5	1.9	1.4	3.4	13.9		5.0	40.0	125	435	294	278	667	278	485	154			
													b			1.5		1.9		2.1		2.5		3.0															
													c			4.5		3.6		3.2		4.8		10.2															
													13									1																	
													a	1.0	13.9	12.7	12.7	13.9	9.9	10.5	14.5	9.7	25.0	16.7	7.0														
													b			1.4		1.9		2.2		2.5		2.9															
													c			17.8		26.4		23.1		24.3		48.4															
7	*21/2	27/2	2/3	6/3	10/3	13/3	16/3	19/3	22/3	25/3				*24/2	26/2	27/2	2/3	6/3	10/3	13/3	16/3	19/3	22/3	25/3			*24/2	26/2	22/7	2/3	6/3	10/3	13/3	16/3	19/3	25/3	30/3		
(1915)	9.4	7.4	10.4	8.3	6.8	7.3	6.9	7 3	6.9	9.3			a	<2	14.6	13.4	12.5	11.2	5.8	7.3	7.6	7.1	7.5	4.9			11.5	14.0	14.4	<1	0.9	1.0	1.0	4.7	9.7	6.2	9.7		
													b				1.4		2.0		2.4		2.8																
													c				17.5		11.6		18.2		21.0																

TABLE 1—*Continued*

D. Anti-saponin

NUMBER OF KID	MOTHER'S SERUM															KID'S SERUM																MILK
12x13	12/2	25/2	*27/2	3/3	5/3	7/3	11/3	14/3	17/3	21/3	28/3	2/4	7/4	11/4	16/4		*27/2	28/2	2/3	5/3	7/3	11/3	14/3	17/3	21/3	25/3	28/3	2/4	7/4	11/4	16/4	27/2–16/4
(1914)	3 6	3.1	2.6	2.5	2.8	3.6	3.3	5.0	3.6	3.3	3.6	3.6	3.7	3.6	4.3	12a	5	5.8	36.0	5.8	4.0	4.0	5.0	5.8	5.8	9.0	8.3	7.6				3/3 0.5
																b		1.1	1.2	1.4	1.5	1.7	1.8	1.9	2.0	2.2	2.4	2.8				7/4 1.0
																c		6.4	43.2	8.1	6.0	6.8	9.0	11.0	11.6	19.8	20.0	21.3				At the remaining dates 0
																13a		5.0	7.8	<5	3.0	4.0	5.0	5.8	5.8	<5	4.5	9.0	6.7	5.4	8.7	
																b		1.1	1.3		1.5	1.7	1.7	1.9	2.1		2.5	2.5	2.7	3.0	3.3	
																c		5.5	10.1		4.5	6.8	8.5	11.0	12.2		11.3	22.5	18.1	16.2	28.7	
15	*1/3	2/3	4/3	7/3	11/3	14/3	17/3	21/3	25/3	28/3	1/4	6/4					*1/3	2/3	4/3	7/3	14/3	17/3	21/3	25/3	28/3	1/4	6/4					1/3–6/4
	8.0	8.3	10.4	8.4	7.5	8.4	10.6	11.5	12	11.4	11.9	12.8				a	11.0	18.5	18.5	19.0	18.5	16.0	16.5	13.0	17.0	17.0	18.5					2/3<1
																b			1.2		1.3	1.6	1.8	2.0	2.2	2.3	2.5					At the remaining dates
																c			22.2		24.1	25.6	29.7	26.0	37.4	39.1	41.3					<1
6	*21/2	27/2	2/3	6/3	10/3	15/3	22/3										*21/2	23/2	27/2	2/3	6/3	10/3	15/3	22/3								21/2–22/3
(1915)	2.0	1.7	1.9	2.1	2.5	2.5	2.5									a		5.0	2.7	2.5	2.7	3.3	2.5	5.5								10/3 0.5 19/3<0.5
																b		1.1	1.4	1.4		1.8										At the remaining dates 0
																c		5.5	3.8	3.5		5.9										
12x13	*25/2	27/2	2/3	6/3	10/3	13/3	16/3	19/3	22/3	25/3	30/3	3/4					*25/2	27/2	2/3	6/3	10/3	13/3	16/3	19/3	22/3	25/3	30/3					25/2–30/3
(1915)	2.6	2.5	2.6	3.0	3.2	3.2	5.0	3.0	3.2	4.0	4.0	3.0				12a	0	11.1	6.2	2.7	2.3	4.5	6.7	11.5	5.0	5.0	4.5					Everywhere 0
																b			1.5		1.9		2.1		2.5		3.0					
																c			9.3		4.4		14.1		12.5		13.5					
																13a	4.0	12.0	5.4	2.7	4.0	5.0	5.0	8.3	10.0	10.5	11.0					
																b			1.4		1.9		2.2		2.5		2.9					
																c			7.6		7.6		11.0		25.0		31.9					

The curves for kids 12 and 13 (1915) are diagrammatically represented. It appears that their increases and decreases are concomitant until toward the end of the observations, when this relationship is disturbed; that is at the time when the kids take nourishment other than the mother's milk.

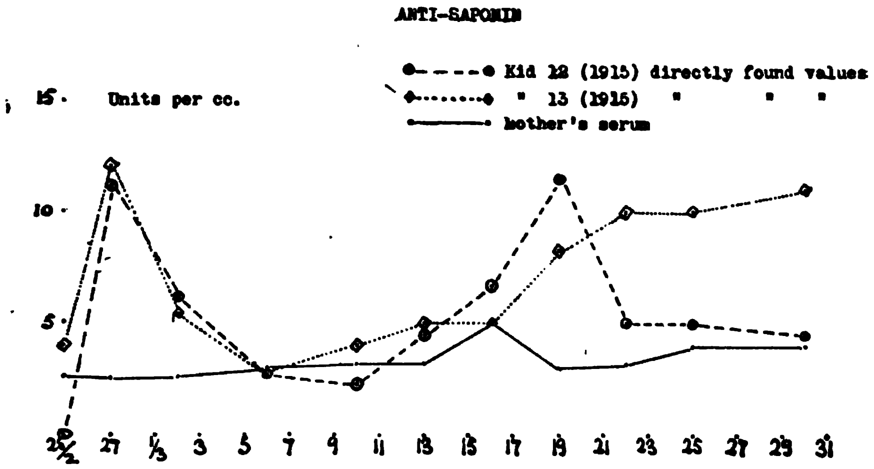

Chart 2

Finally the saponin-neutralizing power of the milk proved slight and offered no support for the supposition that this food influenced the content of these bodies in the blood of the kids.

REFERENCES

(1) Schenk, F.: Monatsschrift fur Geburtshulfe und Gynaekologie, 1903.
(2) Polano, O.: Habilitationsschrift Wurzberg, 1904.
(3) Ransom, F.: Deutsche med. Wochenschrift, 1901, **27**, 194.
(4) Madsen, Th., and Noguchi, H.: Oversigt over det danske Videnskabernes Sekskabs Forhandlingar, 1904, Nr. 6, 457.

A SEROLOGICAL STUDY OF CHOLERA IMMUNITY

I. AGGLUTININ

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The relation of serum proteins to antibodies has been the subject of much investigation in the past, and yet there are a number of points which we do not fully understand. The earliest research published in the literature on the subject is by Widal and Sicard (15) in 1897, who concluded that agglutinin is precipitated together with globulin when the immunized serum is treated with magnesium sulphate. In 1899 Winterberg (16) investigated the action of various protein precipitants and found that agglutinin is almost completely precipitated by sodium sulphate and less completely by magnesium sulphate, ammonium sulphate, sodium acetate and sodium nitrate, while only slightly affected by sodium chloride and potassium chloride. He further stated that animal membranes are impermeable to agglutinin, and that when agglutinin is subjected to dialysis lasting more than a month, its loss seldom exceeds 10 per cent.

Again E. P. Pick (10, 11) came to the conclusion, as may be seen in the table 1, that the typhoid agglutinin obtained from the immunized horse is contained in pseudoglobulin, that in the immune serum obtained from the goat, rabbit and seal agglutinin exists in euglobulin, and that the cholera agglutinin, contrary to the typhoid agglutinin, is contained in euglobulin and never found in fibrinoglobulin or pseudoglobulin.

It was Gibson and Collins in 1907 (3) who stated that their results disagreed with those of Pick, but it is now generally agreed that only the typhoid agglutinin from the horse is found in pseudoglobulin, which is the only exception to the general rule that it is contained in euglobulin.

I shall briefly review the results of investigations by the above workers and others, which I have repeated several times and carefully examined, in order to determine wherein their results disagree. E. P. Pick (10), adopting Hofmeister's method of classification, regarded euglobulin as being precipitated by the addition of one-third saturated ammonium sulphate, and pseudoglobulin by the addition of an equal volume of saturated solution of the same salt to its filtrate. Gibson and Collins thought that the precipitate which they obtained by the addition of 3.4 saturation of ammonium sulphate was euglobulin, while its filtrate was pseudoglobulin. Following the numerous researches of Weyl (14), Panum (9), Kühne (7), Burckhardt (1), Pohl (12), Kauder (5) and others, Marcus (8) distinguished in the serum the water-soluble and water insoluble proteins; while Hofmeister and Pick (10, 11) discovered that the water-insoluble protein can be precipitated by 2.8 to 3.6 saturation of ammonium sulphate and the water-soluble protein by 3.6 to 4.4 saturation of the same salt, calling the former euglobulin and the latter pseudoglobulin. Again, Porges and Sapiro (13) classified the serum proteins into three groups, each group having the precipitation limits by ammonium sulphate at three distinct saturations, namely, 2.8 to 3.6, 3.3 to 4.2 and 4.0 to 4.6; and each protein body is thought to contain soluble proteins. Moreover, Freund and Joachim (2), repeating the experiments of E. P. Pick, found that both euglobulin and pseudoglobulin contain soluble as well as insoluble protein bodies.

TABLE 1*

AGGLUTININ	ANIMAL	FIBRINO-GLOBULIN	EUGLOBULIN	PSEUDOGLOBULIN	ALBUMIN
Typhoid	Horse	0	Trace	Nearly all	0
	Goat	0	All	0	0
	Rabbit	0	All	0	0
Cholera	Seal	0	All	0	0
	Horse	0	All	0	0
	Goat	0	All	0	0

* Pick: Handb. von. Kraus-Levaditi, Bd. I, p. 331.

On the basis of the above experimental data, Hammarsten (4) states, in his recent text-book on physiological chemistry, that the classification of sera on the basis of water-soluble and water-insoluble proteins is incomplete, and that the separation of serum proteins by means of ammonium sulphate is far from satisfactory. Accordingly, I diluted the serum five times with distilled water in order to minimize the above interaction of serum protein bodies, and yet within the limits of non-interference with the examination of possible agglutination reaction, and drew a distinction between the first, the second and the third euglobulins, all of which were free from potential pseudoglobulin and were precipitated at various precipitation points. Thus the first euglobulin was precipitated at the highest point, namely, 3.3 saturation, the second and the third at 3.4 and 3.6 saturations respectively. Likewise pseudoglobulin is divided into three groups as follows: the precipitate which is obtained by half saturating the filtrate which remains after the complete separation of euglobulin by means of 3.6 saturation of ammonium sulphate is called the first pseudoglobulin, while the precipitate obtained by half saturating the filtrates after the separation of the first and the second euglobulin by means of 3.3 and 3.4 saturations are regarded as the third and the second pseudoglobulin respectively.

EXPERIMENTAL DATA

1. Preparation of saturated aqueous solution of ammonium sulphate

Ammonium sulphate is at first dissolved in appropriate quantity of distilled water until fully saturated, and any iron present is precipitated by passing hydrogen sulphide gas. It is evaporated and made into a heat saturated condition, then cooled to recrystallization by placing in running water. This recrystallization method is repeated six times. The crystals of ammonium sulphate thus purified are used in making the saturated solution for the present experiments, by taking about 20 per cent in excess of 770 grams of the purified crystals and dissolving in 1 liter of heated distilled water. This is left at room temperature until no more crystal is seen to go into the solution. The clear supernatant portion is used as saturated solution.

2. *Separation of the serum*

The immune serum described in the present experiments has been obtained from the blood of the jugular vein by letting the corpuscular elements settle at the bottom of the containing vessels after standing for twenty-four hours in a cool place.

3. *Separation of serum protein bodies*

The serum, which has previously been diluted five times its volume with distilled water, is placed in three Becher glasses (50 cc. each), and while stirring the saturated aqueous solution of ammonium sulphate is added gradually in different quantities, namely, 24.62 cc., 24.76 cc., and 28.12 cc. respectively. The precipitates formed in the above treatment are the three varieties of euglobulin; 3.3 saturation being the first, 3.4 saturation the second and the 3.6 saturation the third euglobulin. These are filtered after three hours, and the process of filtration is repeated until the filtrates are perfectly clear. Each of the above precipitates is washed with 3.3, 3.4 and 3.6 saturation of ammonium sulphate solution repeatedly until the solution becomes completely negative when treated with Spiegler's solution. Each filtrate is then centrifuged and 37.31 cc., 37.88 cc., and 39.06 cc. of its clear supernatant liquid is treated with 12.49 cc., 12.12 cc., and 10.94 cc., of saturated aqueous solution of ammonium sulphate by adding the solution gradually while stirring, and the precipitates (the third, the second and the first pseudoglobulins) are filtered repeatedly until perfectly clear. The precipitates thus obtained are dried thoroughly by pressing between the filter papers, and then redissolved in distilled water, making up to the original volume.

4. *Examination for agglutination reactions*

Each of the immune sera and the separated protein bodies was diluted to twice the volume with sterile saline. The bacillary emulsion was made by adding 10 cc. of sterile saline to one slant of eighteen-hour culture in an agglutination tube. Two drops of each emulsion and serum protein were put in a tube and incubated first at 37°C. for two hours and then at room temperature for twenty-four hours and examined.

HORSE SERUM IMMUNIZED AGAINST CHOLERA

E. P. Pick precipitated euglobulin by the direct one-third saturation with ammonium sulphate without diluting the strongly positive horse serum immunized against cholera having the agglutination value of 10,000. He also precipitated pseudoglobulin by half saturating the filtrate with ammonium sulphate.

TABLE 2*

	SERUM DILUTION					
	20	200	2000	4000	6000	10,000
Euglobulin	+	+	+	+	+	+
Pseudoglobulin	+	+	–	–	–	–

* E. P. Pick: Hofmeister's Beiträge, Bd. I, p. 378.

TABLE 3*

	SERUM DILUTION				
	50	100	200	500	1000
Euglobulin	+	+	–	–	–
Pseudoglobulin	++++	++++	+++	+++	+

* Gibson and Collins: Journal of Biolog. Chemistry, vol. III, p. 246.

His results are summarized in table 2, in which it is clearly brought out that while euglobulin retains the original agglutination value of 10,000, pseudoglobulin shows a titer of only 200, and yet he explains that even this small value may probably be due to some substance which escaped through the filter paper and thus it may be said that pseudoglobulin contains no agglutinin. But the results obtained by Gibson and Collins are entirely opposite. They precipitated euglobulin at 3.4 saturation with ammonium sulphate from the serum having the agglutination value of 1000, and its filtrate they regarded as being pseudoglobulin. They found that while euglobulin was positive only

TABLE 4

Agglutination values of protein bodies in the serum of horse A

	DILUTION											
	1/640	1/960	1/1280	1/1920	1/2560	1/3840	1/5120	1/7680	1/10240	1/15360	1/20480	1/30720
Serum	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	−
Euglobulin I	+++	+++	+++	++	+	±	−					
Euglobulin II	+++	+++	+++	+++	+	+	−					
Euglobulin III	+++	+++	+++	+++	++	++	+	±	−			
Pseudoglobulin I	+++	+++	+++	+++	+++	+++	+++	++	+	−		
Pseudoglobulin II	+++	+++	+++	+++	+++	+++	+++	++	+	−		
Pseudoglobulin III	+++	+++	+++	+++	+++	+++	+++	+++	++	+	−	

TABLE 5

Agglutination values of protein bodies in the serum of horse B

	DILUTION										
	1/1280	1/1920	1/2560	1/3840	1/5120	1/7680	1/10240	1/15360	1/20480	1/30720	1/40960
Serum	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	+
Euglobulin I	+++	+++	+++	++	++	+	−				
Euglobulin II	+++	+++	+++	++	++	+	±	−			
Euglobulin III	+++	+++	+++	+++	++	++	±	−			
Pseudoglobulin I	+++	+++	+++	+++	+++	+++	+++	++	+	−	
Pseudoglobulin II	+++	+++	+++	+++	+++	+++	+++	++	+	−	
Pseudoglobulin III	+++	+++	+++	+++	+++	+++	+++	+++	++	+	−

to 100, pseudoglobulin was positive up to 1000. The results of my own study of the agglutination values of the protein bodies, which have been separated according to the method already described, may be summarized as shown in tables 4 and 5.

From these two tables it is clear that the agglutinin in the serum of the horse immunized against cholera not only exists in the euglobulin fraction, as brought out by Pick, but is present for the greater part in the pseudoglobulin fraction as Gibson and Collins have shown.

The reason why these investigators obtained results so much at variance with one another may be inferred from the fact that, while Pick used the serum without diluting, Gibson and Collins diluted it twice its volume. The two questions which we must at once seek to answer are: (1) Whether or not the presence of ammonium sulphate hindered the agglutination reaction, and (2) whether or not the dilution of the serum had anything to do with the results. In order to test these points, the following experiments were made:

1. Does the presence of ammonium sulphate hinder the agglutination reaction?

The serum was diluted five times (100 cc. of serum and 400 cc. of distilled water) and 250 cc. of saturated ammonium sulphate were added, making the degree of saturation of the entire mixture one-third. The filtrate was again half-saturated with the saturated solution of ammonium sulphate. The precipitates thus obtained in both cases were dialyzed for three weeks. After proving the absence of sulphuric acid and ammonia by means of barium chloride and Nessler's reagent respectively, the agglutination reactions were tested and the results compared with those obtained with the undialyzed fluid, as shown in table 6.

This table shows that ammonium sulphate, which may be present after the manipulation as stated in the introductory paragraphs, hinders in no way the agglutination reactions.

TABLE 6

	DILUTION										
	1/960	1/1280	1/1920	1/2560	1/3840	1/5120	1/7680	1/10240	1/15360	1/20480	1/30720
Horse serum.....	+++	+++	+++	+++	+++	+++	+++	+++	++	+	–
Dialyzed euglobulin.......	+++	+++	++	+	±	–					
Dialyzed pseudoglobulin	+++	+++	+++	+++	+++	+++	+++	++	±	–	
Uudialyzed euglobulin.......	+++	+++	++	+	+	–					
Undialyzed pseudoglobulin	+++	+++	+++	+++	+++	+++	+++	++	+	–	

2. Do the differences in results depend upon whether or not the serum was diluted?

Ten cubic centimeters of the serum were made 3.6 saturated without diluting by adding 5.62 cc. of saturated aqueous solution of ammonium sulphate. Again, as before, 3.6 saturation was made from the serum diluted five and ten times respectively. After three hours the precipitates in the above mixtures were filtered, and the agglutination values of both the precipitates and the filtrates were compared as shown in the next table (7). The filtrates were in each case made transparent by repeated filtration and the precipitates were washed with 3.6 saturated solution of the salt, and repeated until the latter gave no reaction with the Spiegel's solution. The precipitate was then dried by pressing between the filter papers and the solution was made by adding distilled water, restoring the original volume of the serum.

According to table 7, although the amount of precipitate is greater in the undiluted serum than in the diluted, yet in no case did the agglutinin come down exclusively in one fraction as reported by E. P. Pick.

Thus it appears that the above stated questions are in both cases negatively answered, and hence the above process and results may be considered normal. I further endeavored to determine at what saturation the agglutinin began to come down and at what point is it completely precipitated. When this is determined we shall be able to say whether or not it is contained in fibrinoglobulin and albumin as reported by E. P. Pick. The following experiments were carried out.

TABLE 7

	DILUTION									
	1/960	1/1280	1/1920	1/2560	1/3840	1/5120	1/7680	1/10240	1/15360	1/20480
Horse serum	+++	+++	+++	+++	+++	+++	+++	+++	++	+
Undiluted										
Precipitate	+++	+++	+++	+++	++	+	+			
Filtrate	3	3	3	3	3	2	1			
5 volume dilution										
Precipitate	+++	+++	+++	++	++	+	±			
Filtrate	3	3	3	3	3	3	2	1		
10 volume dilution										
Precipitate	+++	+++	+++	++	++	+	±			
Filtrate	3	3	3	3	3	3	2	1		

Note: In the table, +++, ++, +, — indicate the degree of agglutination with the precipitate as in table 4. The figures, 3, 2, 1 designate the agglutination reaction with the filtrate, — strong, medium and weak.

THE RELATION OF AGGLUTININ TO THE PRECIPITATION POINT OF SERUM PROTEINS

The serum was treated with ammonium sulphate beginning with 2.4 saturation and gradually increasing the amount of salt up to half saturation. The precipitate and the filtrate were examined as to their agglutination values, which may be tabulated as shown in tables 8 and 9.

We were not able to examine the precipitate obtained at 2.6 saturation with ammonium sulphate because of its small amount, but it may be said to contain some agglutinin, judged by the fact that the precipitate obtained at 2.8 saturation gave a positive reaction. In other words, the agglutinin begins to come down with the precipitate by ammonium sulphate and the precipita-

TABLE 8

NUMBER	SERUM	H_2O	$(NH_4)_2SO_4$	DILUTION 1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/960	1/1280	1/1920	1/2560	1/3840	1/5120	1/7680	1/10240	1/15360	1/20480	1/30720
1	2.0	5.6	2.4		Clear																
2	2.0	5.4	2.6		Turbid																
3	2.0	5.2	2.8	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	++ 3	++ 3	+ 3	± 3	− 3	3	3	3	2	1		
4	2.0	5.0	3.0	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	++ 3	++ 3	+ 3	− 3	3	3	3	2	1		
5	2.0	4.8	3.2	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	++ 3	++ 3	+ 3	+ 3	− 3	3	2	1		
6	2.0	4.6	3.4	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	++ 3	+ 3	+ 3	− 3	2	1			
7	2.3	4.4	3.6	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	++ 3	++ 3	+ 3	± 2	1			
8	2.0	4.2	3.8	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	++ 3	++ 2	− 1	+			
9	2.0	4.0	4.0	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 2	++ 1	++	+	−		

10	2.0	3.8	4.2	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 2	+++ 1	+++	+++	++	++	+	–	
11	2.0	3.6	4.4	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 2	+++ 1	+++ 1	+++	+++	+++	+++	+++	+++	++	+	–	
12	2.0	3.4	4.6	+++ 3	+++ 3	+++ 2	+++ 2	+++ 1	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	±	–
13	2.0	3.2	4.8	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	+	–
14	2.0	3.0	5.0	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	+	–
Control serum . . .				+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	+	–

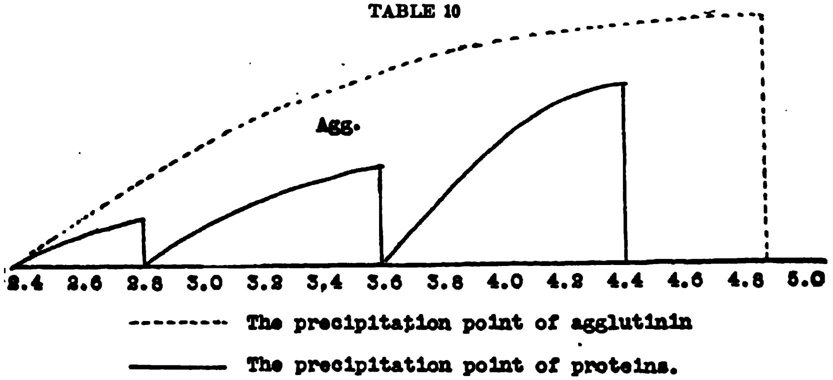
TABLE 9

NUMBER	SERUM	H₂O	$(NH_4)_2SO_4$	DILUTION																			
				1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/960	1/1280	1/1920	1/2560	1/3840	1/5120	1/7680	1/10240	1/15360	1/20480	1/30720	1/40760	1/61440
1	2.0	5.6	2.4	Opalescent																			
2	2.0	5.4	2.6	Turbid																			
3	2.0	5.2	2.8	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	++ 3	+ 3	+ 3	– 3	3	3	3	3	2	1	1	
4	2.0	5.0	3.0	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	++ 3	++ 3	+ 3	3	3	3	3	2	1		
5	2.0	4.8	3.2	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	++ 3	++ 3	+ 3	3	3	3	2	1		
6	2.0	4.6	3.4	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	++ 3	++ 3	+ 3	± 3	2	1			
7	2.0	4.4	3.6	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	++ 3	++ 3	± 3	2	1			
8	2.0	4.2	3.8	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	++ 3	++ 2	+ 1				
9	2.0	4.0	4.0	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 2	+++ 1	++	+	+			

10	2.0	3.8	4.2	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 2	+++ 1	+++ 1	+++	+++	+++	+++	++	++	+	−	
11	2.0	3.6	4.4	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 2	+++ 1	+++	+++	+++	+++	+++	+++	+++	++	+	±	
12	2.0	3.4	4.6	+++ 3	+++ 3	+++ 3	+++ 2	+++ 1	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	+	±	
13	2.0	3.2	4.8	+++ 2	+++ 1	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	+	±
14	2.0	3.0	5.0	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	+	±
Control serum . . .				+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	+	±

tion is complete at 4.8 saturation. As to the agglutinin present in the filtrate, we may say that in general the sum of the agglutination values in both the filtrate and the precipitate obtained at 2.8 to 3.8 saturation equals the original agglutination value of the serum, but we also find that the agglutination value of the filtrate at a saturation greater than 4.0 is markedly decreased. This may be due to the fact that the presence of ammonium sulphate to an extent greater than 4.0 saturation hinders the agglutination reaction. The relation of the agglutinin to the protein bodies which precipitate with the saturated solution of ammonium sulphate may be expressed diagrammatically as shown in table 10.

TABLE 10



The classification of various protein bodies, according to Hofmeister and Pick, is as follows:

Fibrinoglobulin	2.8 saturation and lower
Euglobulin	2.8–3.6 saturation
Pseudoglobulin	3.6–4.4 saturation

Thus, contrary to the opinion of E. P. Pick, agglutinin is present in fibrinoglobulin, while it is absent in albumin.

RELATION OF SERUM PROTEINS OBTAINED FROM DIFFERENT ANIMALS TO AGGLUTINATION

Does the above relationship of agglutinin to serum proteins obtain in the protein bodies of other types of immunized animals, or, as E. P. Pick has stated, is there a difference according to the species? This is an interesting question both from the standpoint of biology and from that of immunology, and the attempt has been made to determine this point in the following experiments with different animals.

Goat serum immunized against cholera

E. P. Pick examined goat serum immunized against cholera, which had the agglutination value of 2000, in the same manner as he did the horse serum, and demonstrated the large amount of agglutinin in euglobulin as is shown in table 11.

TABLE 11*

	DILUTION											
	1/20	1/40	1/80	1/100	1/200	1/500	1/1000	1/1200	1/1400	1/1600	1/1800	1/2000
Euglobulin	+	+	+	+	+	+	+	+	+	+	+	−
Pseudoglobulin	+	+	+	−								

* E. P. Pick: Hofmeisters Beiträge, Bd. I, p. 380.

TABLE 12*

	DILUTION				
	1/50	1/100	1/200	1/500	1/1000
Serum	++++	++++	++++	++++	++++
Euglobulin	++	+	+		
Pseudoglobulin	++++	++++	++++	++	±

* Gibson and Collins: Journ. of Biolog. Chemistry, Vol. III, p. 243.

But Gibson and Collins reported results quite contrary to those of Pick, as is shown in the copy of one of their tables (table 12).

I separated the goat serum immunized against cholera in the same manner as I dealt with the horse serum and obtained the results detailed in table 13.

Thus, as there seemed to be no distinct difference as regards agglutinin content between euglobulin and pseudoglobulin in

TABLE 13

Agglutination values of proteins from goat serum

	DILUTION												
	1/240	1/320	1/480	1/640	1/960	1/1280	1/1920	1/2560	1/3840	1/5120	1/7680	1/10240	1/15360
Serum	+++	+++	+++	+++	+++	+++	+++	+++	++	++	+	±	−
Euglobulin I	+++	+++	++	++	+								
Euglobulin II	+++	+++	++	++	+	±							
Euglobulin III	+++	+++	+++	++	++	+							
Pseudoglobulin I	+++	+++	+++	+++	+++	++	++	+					
Pseudoglobulin II	+++	+++	+++	+++	+++	+++	+++	++	++	+			
Pseudoglobulin III	+++	+++	+++	+++	+++	+++	+++	++	++	+			

TABLE 14

NUMBER	SERUM	H_2O	$(NH_4)_2SO_4$	DILUTION															
				1/10	1/20	1/40	1/80	1/120	1/160	1/240	1/320	1/480	1/640	1/960	1/1280	1/1920	1/2560	1/3840	1/5120	1/7680	1/10240
1	2.0	5.6	2.4	Opalescent																	
2	2.0	5.4	2.6	Turbid																	
3	2.0	5.2	2.8	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	++ 3	++ 3	+ 3	3	3	3	3	3	3	2	2	1	
4	2.0	5.0	3.0	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	++ 3	++ 3	+ 3	± 3	3	3	3	3	2	1	1	

5	2.0	4.8	3.2	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	++ 3	++ 3	+ 3	± 3	3	3	2	2	1		
6	2.0	4.6	3.4	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	++ 3	++ 3	+ 3	± 3	3	2	2	1		
7	2.0	4.4	3.6	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	++ 3	++ 3	+ 3	2	2	1			
8	2.0	4.2	3.8	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	++ 3	++ 2	+ 1	± 1				
9	2.0	4.0	4.0	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 2	+++ 1	+++	+++	++	++	+				
10	2.0	3.8	4.2	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 2	+++ 1	+++	+++	+++	+++	+++	++	++	+			
11	2.0	3.6	4.4	+++ 3	+++ 3	+++ 3	+++ 2	+++ 1	+++	+++	+++	+++	+++	+++	+++	+++	++	++	+		
12	2.0	3.4	4.6	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	+	−
13	2.0	3.2	4.8	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	+	±
Control serum......				+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	+	±

TABLE 15

NUMBER	SERUM	H_2O	$(NH_4)_2SO_4$	DILUTION 1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/960	1/1280	1/1920	1/2560	1/3840	1/5120	1/7680	1/10240	1/15360
1	2.0	5.6	2.4	Clear															
2	2.0	5.4	2.6	Opalescent															
3	2.0	5.2	2.8	Turbid															
4	2.0	5.0	3.0	Strongly turbid															
5	2.0	4.8	3.2	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	++ 3	++ 3	+ 3	3	3	3	2	1		
6	2.0	4.6	3.4	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	++ 3	++ 3	+ 3	3	2	2	1		
7	2.0	4.4	3.6	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	++ 3	++ 3	+ 3	± 2	1			
8	2.0	4.2	3.8	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	++ 2	++ 1	+ 1				
9	2.0	4.0	4.0	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 2	+++ 2	+++ 1	++	+				
10	2.0	3.8	4.2	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 2	+++ 1	+++	+++	+++	+++	++	++	+		

11	2.0	3.6	4.4	+++ 3	+++ 3	+++ 3	+++ 2	+++ 1	+++ 1	+++	+++	+++	+++	+++	+++	++	++	+	
12	2.0	3.4	4.6	+++ 3	+++ 2	+++ 1	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	+	–
13	2.0	3.2	4.8	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	+
14	2.0	3.0	5.0	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	+
Control serum.....				+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	+

the goat serum, the experiments in fractional precipitation of the proteins were repeated with the results shown in tables 14 and 15.

Thus in the tables 14 and 15, we learn that, in the goat serum immunized against cholera, there is a relationship between the precipitation point of the agglutinin and that of euglobulin with ammonium sulphate similar to that observed for immune horse serum.

Rabbit serum immunized against cholera

Although we do not find any mention of rabbit serum immunized against cholera in the report of E. P. Pick, we do find table 16 representing the result of an examination by Gibson and Collins of such serum carried out in the same manner as that in which they separated the horse serum.

TABLE 16*

	DILUTION					
	1/50	1/100	1/200	1/300	1/1000	1/2000
Pseudoglobulin.............	++++	++++	++++	++++	++++	+
Euglobulin.................	+	−	−	−	−	−

* Gibson and Collins: Journ. of Biolog. Chemistry, Vol. III, p. 241.

My own results with rabbit serum immunized against cholera having the following agglutination value is shown in table 17.

Thus the agglutinin in the rabbit serum immunized against cholera does not exist either in euglobulin or in pseudoglobulin exclusively. I have repeated with the rabbit serum the experiments conducted with horse and goat serum and obtained the results in table 18.

In looking over the above table, we note that there is no distinction in the precipitation points of agglutinin in the rabbit serum immunized against cholera from those of the horse or goat sera immunized against cholera.

TABLE 17

	DILUTION												
	1/160	1/240	1/320	1/480	1/640	1/960	1/1280	1/1920	1/2560	1/3840	1/5120	1/7680	1/10240
Serum	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	+	+
Euglobulin I	+++	+++	++	++	+	±							
Euglobulin II	+++	+++	++	++	+	+							
Euglobulin III	+++	+++	+++	++	++	+	+						
Pseudoglobulin I	+++	+++	+++	+++	+++	+++	+++	++	++	+			
Pseudoglobulin II	+++	+++	+++	+++	+++	+++	+++	++	++	+	+		
Pseudoglobulin III	+++	+++	+++	+++	+++	+++	+++	++	++	+	+		

TABLE 18

NUMBER	SERUM	H_2O	$(NH_4)_2SO_4$	DILUTION																
				1/10	1/20	1/40	1/80	1/120	1/160	1/240	1/320	1/480	1/640	1/960	1/1280	1/1920	1/2560	1/3840	1/5120	1/7680	1/10240
1	2.0	5.6	2.4	Clear																	
2	2.0	5.4	2.6	Opalescent																	
3	2.0	5.2	2.8	Turbid																	
4	2.0	5.0	3.0	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	++ 3	++ 3	+ 3	3	3	3	3	3	3	2	1	1	
5	2.0	4.8	3.2	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	++ 3	+ 3	+ 3	3	3	3	3	2	1	1		
6	2.0	4.6	3.4	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	++ 3	+ 3	+ 3	3	3	2	2	1			
7	2.0	4.4	3.6	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	++ 3	++ 3	+ 3	+ 3	2	2	1			
8	2.0	4.2	3.8	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	++ 3	++ 2	+ 2	± 1					
9	2.0	4.0	4.0	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 3	+++ 2	+++ 1	+++ 1	+++	++	++	+	+				
10	2.0	3.8	4.2	+++ 3	+++ 3	+++ 3	+++ 3	+++ 2	+++ 1	+++	+++	+++	+++	+++	++	++	+	±			

11	2.0	3.6	4.4	+++ 3	+++ 3	+++ 3	+++ 2	+++ 1	+++	+++	+++	+++	+++	+++	+++	++	++	+	+		
12	2.0	3.4	4.6	+++ 3	+++ 3	+++ 2	+++ 1	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	+	+	
13	2.0	3.2	4.8	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	+	±
Control serum				+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	+	±

CONCLUSION

We have thus far described the outline of various experiments after each of which we added our brief comment. On the basis of these results, we may draw the following conclusions:

1. Agglutinin begins to come down at the time when the serum proteins begin to precipitate by the addition of ammonium sulphate, and is completely precipitated at 4.6 to 4.8 saturation, and therefore,
2. Agglutinin is present in both euglobulin and pseudoglobulin and never in either exclusive of the other.
3. It is present also in fibrinoglobulin, but not in albumin.
4. There is a uniform relationship between the agglutinin and the protein bodies in all animals (horse, goat and rabbit).

The author is deeply grateful to the kind supervision and criticism of Professor Yokote and Dr. Kawamoto, and to the helpful assistance of Drs. Y. Kato and Tamiya.

REFERENCES

(1) Burckhardt, A. E.: Arch. f. exp. Path. u. Pharm., 1883, **16**, 322.
(2) Freund, E., u. Joachim, J.: Zeits. f. physiol. Chem., 1902, **36**, 407.
(3) Gibson, R. B., and Collins, K. R.: Journ. of Biol. Chem., 1907, **3**, 233:
(4) Hammarsten, O.: Lehrbuch d. phys. Chem., Bd. 3.
(5) Kauder, G.: Arch. f. exp. Path. u. Pharm., 1886, **20**, 411.
(6) Kolle-Wassermann: Handb. d. path. Mikrorg., Bd. 2 (1913).
(7) Kühne, W.: Lehrbuch der physiol. Chemie, Leipzig, 1866–68.
(8) Marcus, E.: Zeits. f. physiol. Chem., 1899, **28**, 559.
(9) Panum, P.: Virchow's Archiv, 1851, **3**, 251; 1852, **4**, 17, 419.
(10) Pick, E. P.: Hofmeister's Beiträge, 1901, **1**, 378.
(11) Pick, E. P.: Handb. von Kraus-Levaditi, 1908, 1, 331.
(12) Pohl, J.: Arch. f. exp. Path. u. Pharm., 1886, **20**, 426.
(13) Porges, O., u. Sapiro, K.: Hofmeister's Beiträge, 1903, **3**, 277.
(14) Weyl, Th.: Beiträge zur Kenntnis thierischer und pflanzlicher Eiseisskörper.
(15) Widal, F., u. Sicard, A.: Ann. de l'inst. Past., 1897, **11**, 353.
(16) Winterberg, H.: Zeits. f. Hyg., 1899, **32**, 375.

THE VALUE OF THE INTRA-PALPEBRAL MALLEIN TEST IN THE DIAGNOSIS OF GLANDERS

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While on duty in the Base Laboratory, Base Section No. 1, American Expeditionary Forces, an opportunity was offered to study the relation of the intra-palpebral mallein test, as advocated by the French veterinarians upon horses and mules, to the serological findings.

The work being reported was conducted during the months of February and March, 1919, with the assistance of the staff of Veterinary Hospital No. 9, St. Nazaire. It consisted in a study of the agglutination and complement fixation reactions of the sera of 94 horses and 8 mules; animals which gave slight but not definitely diagnostic intra-palpebral mallein reactions. Similar reactions were performed upon a control series of 51 horses, all of which gave negative intra-palpebral mallein reactions.

The 94 horses and 8 mules were bled on February 7, 1919, and subsequently "malleined" on February 9, 1919, the readings from that test being the ones recorded in table 1. All of these animals had been previously "malleined" on January 22, 1919. The 51 controls had been "malleined" on February 9, 1919 and were bled on February 20, 1919. The intervals elapsing between the last previous malleination and the bleeding, sixteen days in the case of the suspicious animals, and eleven days in the controls, is subject to criticism, we think, as regards excluding the presence of specific agglutinins or complement fixing bodies in the sera at the time of bleeding.

The complement fixation reactions were performed as directed by Memorandum No. 34, Office of the Chief Surgeon, Division of Laboratories and Infectious Diseases, American Expeditionary Forces, mule sera being inactivated at 62°C. for half an hour.

TABLE 1

Horse sera

SERIAL NUMBER	NUMBER OF HORSE	MALLEINATION, HOURS AFTER TEST			COMPLEMENT FIXATION		AGGLUTINATION, SERIES 1, FEBRUARY 18, 1919			AGGLUTINATION, SERIES 2, FEBRUARY 24, 1919					
		24	28	32	No. 1, February 14, 1919	No. 2, February 17, 1919	$\frac{1}{400}$	$\frac{1}{600}$	$\frac{1}{900}$	$\frac{1}{600}$	$\frac{1}{900}$	$\frac{1}{1200}$	$\frac{1}{1800}$	$\frac{1}{2400}$	$\frac{1}{3600}$
5	1753	2	2	2	+	+	++	++	+	++	++	−	−	−	−
6	1711	2	2	2	++	++	++	++	−	++	+	−	−	−	−
7	1759	2	2	2	+	+	++	+	−	+	−	−	−		
10	1762	0	2	2	+	+	++	+	±	+	−	−	−	−	−
12	1779	1	2	2	+	+	++	+	−	−	−	−	−	−	−
13	1369	1	1	1	±	+	±	−	−	−	−	−	−		
14	1327	1	1	1	−	±	++	−	−	−	−	−	−		
15	227	0	2	2	−	−	±	−	−	−	−	−	−		
19	1396	1	1	1	−	−	−	−	−	−	−	−	−		
20	946	1	1	1	+	+	++	+	−	+	−	−	−		
22	393	1	1	1	++	++	++	++	+	++	+	+	−	−	−
23	381	1	1	1	−	±	+	−	−	−	−	−	−		
24	1317	1	1	1	++	+	++	+	+	+	+	−	−	−	−
25	1325	1	1	1	+	+	++	+	±	+	−	−	−	−	−
26	380	1	1	1	+	+	+	±	−	−	−	−	−		
27	1275	1	1	1	+	±	++	++	−	++	±	−	−	−	−
28	2342	1	1	1	±	++	+	±	±	±	−	−	−	−	−
29	2377	1	1	1	++	++	−	−	−	−	−	−	−		
30	2603	1	1	1	+	+	++	++	++	++	++	±	−	−	−
31	2531	1	1	1	±	±	+	+	−	−	−	−	−		
32	2589	1	1	1	+	+	++	++	++	++	++	++	+	−	−
33	2271	1	1	1	+	+	++	++	+	+	+	−	−	−	−
34	320	1	1	1	++	++	++	+	−	+	−	−	−		
35	999	1	1	1	−	−	+	−	−	−	−	−	−		
36	989	1	1	1	+	+	++	++	++	++	+	−	−	−	−
37	1311	1	1	1	+	+	++	++	+	+	±	−	−	−	−
38	1380	1	1	1	−	−	±	−	−	−	−	−	−		
39	2521	1	1	1	±	+	+	−	−	−	−	−	−		
40	2575	1	1	1	+	+	++	++	±	++	−	−	−	−	−
41	1424	0	1	1	±	+	++	+	−	−	−	−	−		
42	385	0	0	1	−	±	+	+	−	−	−	−	−		
43	341	1	1	1	+	±	++	++	−	++	−	−	−		
44	383	1	1	1	+	+	+	±	±	++	−	−	−	−	−
45	332	1	1	1	+	+	+	±	−	−	−	−	−	−	
46	1026	1	1	1	+	±	+	+	±	+	−	−	−	−	−
47	356	1	1	1	+	+	++	++	++	++	+	−	−	−	−
48	366	1	1	1	+	++	++	−	−	−	−	−	−		
49	316	1	1	1	+	+	+	±	−	−	−	−	−		

TABLE 1—*Continued*

SERIAL NUMBER	NUMBER OF HORSE	MALLEINATION, HOURS AFTER TEST			COMPLEMENT FIXATION		AGGLUTINATION, SERIES 1, FEBRUARY 18, 1919			AGGLUTINATION, SERIES 2, FEBRUARY 24, 1919					
		24	28	32	No. 1, February 14, 1919	No. 2, February 17, 1919	1/400	1/600	1/900	1/600	1/900	1/1200	1/1800	1/2400	1/3600
50	303	1	1	1	+	−	++	±	−	+	−	−	−		
51	352	1	1	1	+	+	±	−	−	−	−	−	−		
52	335	1	1	1	+	+	++	+	±	+	−	−	−	−	−
53	1329	1	1	1	±	±	+	±	−	−	−	−	−	−	−
54	370	0	1	1	±	±	+	−	−	−	−	−	−		
55	382	1	1	1	±	+	++	−	−	−	−	−	−		
56	2522	1	1	1	+	+	++	++	−	++	+	−	−	−	−
57	1322	1	1	1	+	+	++	++	−	+	−	−	−	−	−
58	982	1	1	1	+	+	++	++	−	−	−	−	−	−	−
59	821	1	1	1	±	+	++	++	±	++	+	−	−	−	−
60	2593	1	1	1	±	±	++	++	+	++	++	−	−	−	−
61	1011	1	1	1	±	±	+	+	−	−	−	−	−	−	−
62	310	1	1	1	−	±	±	−	−	−	−	−	−		
63	1393	1	1	1	+	±	++	+	±	+	−	−	−	−	−
64	238	1	1	1	++	++	±	−	−	−	−	−	−		
65	935	1	1	1	−	−	−	−	−	−	−	−	−		
66	1320	1	1	1	++	++	++	++	±	++	++	+	±	−	−
67	1308	1	1	1	+	±	++	−	−	−	−	−	−		
68	1305	1	1	1	+	±	++	−	−	−	−	−	−		
69	318	1	1	1	+	+	+	+	+	−	−	−	−	−	−
70	2371	1	1	1	++	++	++	++	++	++	++	++	+	−	−
71	1203	1	1	1	−	±	+	±	−	−	−	−	−		
72	850	1	1	1	+	+	++	++	±	+	−	−	−	−	−
73	857	1	1	2	++	++	++	+	−	±	−	−	−		
74	844	1	1	1	−	±	++	+	±	+	−	−	−	−	−
75	533	0	1	1	+	+	+	−	−	−	−	−	−	−	−
76	632	1	1	1	++	++	++	++	++	++	+	−	−	−	−
77	602	1	1	1	+	±	++	++	+	+	−	−	−	−	−
78	795	1	1	1	−	−	+	±	−	−	−	−	−		
79	114	1	1	1	−	+	±	−	−	−	−	−	−		
80	205	1	1	1	+	+	++	+	−	−	−	−	−		
81	568	1	1	1	++	++	+	+	+	+	−	−	−	−	−
82	565	1	1	1	+	+	++	++	++	+	−	−	−	−	−
83	948	1	1	1	+	±	±	−	−	−	−	−	−		
84	2655	1	1	1	++	+	±	−	−	−	−	−	−		
85	2347	1	1	1	+	+	++	±	−	+	−	−	−		
86	2314	1	1	1	−	−	±	−	−	−	−	−	−		
87	2615	1	1	1	−	+	++	++	±	+	−	−	−	−	−
88	2404	1	1	1	++	++	±	−	−	−	−	−	−		

TABLE 1—*Concluded*

SERIAL NUMBER	NUMBER OF HORSE	MALLEINATION, HOURS AFTER TEST			COMPLEMENT FIXATION		AGGLUTINATION, SERIES 1, FEBRUARY 18, 1919			AGGLUTINATION, SERIES 2, FEBRUARY 24, 1919					
		24	28	32	No. 1, February 14, 1919	No. 2, February 17, 1919	$\frac{1}{400}$	$\frac{1}{600}$	$\frac{1}{900}$	$\frac{1}{600}$	$\frac{1}{900}$	$\frac{1}{1200}$	$\frac{1}{1800}$	$\frac{1}{2400}$	$\frac{1}{3600}$
89	2583	1	1	1	±	+	+	±	−	−	−	−	−		
90	779	1	1	1	+	+	++	−	−	±	−	−	−		
91	700	1	1	1	++	+	++	++	++	+	−	−	−	−	−
92	259	1	1	1	+	+	+	−	−	−	−	−	−		
94	206	1	1	1	+	++	±	−	−	−	−	−	−		
95	771	1	1	1	−	±	+	−	−	−	−	−	−		
96	1277	1	1	1	+	+	++	±	−	−	−	−	−		
98	2581	1	1	1	++	++	++	±	−	−	−	−	−		
100	32	1	1	1	+	+	++	++	±	++	++	++	+	−	−
101	7	1	1	1	±	±	+	−	−	±	−	−	−		
102	c–3	1	1	1	±	±	++	+	−	−	−	−	−		
103	c–20	1	1	1	+	+	+	±	−	−	−	−	−		
104	977	1	1	1	++	++	++	+	−	++	++	−	−		
105	371	1	1	1	+	+	++	++	±	−	−	−	−	−	−
106	362	1	1	1	+	+	++	+	−	−	−	−	−		
107	1252	1	1	1	±	±	++	++	−	±	−	−	−		
108	1353	1	1	1	−	±	++	++	±	−	−	−	−	−	−

TABLE 2

Mule sera

SERIAL NUMBER	NUMBER OF MULE	MALLEINATION, HOURS AFTER TEST			COMPLEMENT FIXATION		AGGLUTINATION, SERIES 1, FEBRUARY 18, 1919			AGGLUTINATION, SERIES 2, FEBRUARY 24, 1919					
		24	28	32	No. 1, February 14, 1919	No. 2, February 17, 1919	$\frac{1}{400}$	$\frac{1}{600}$	$\frac{1}{900}$	$\frac{1}{600}$	$\frac{1}{900}$	$\frac{1}{1200}$	$\frac{1}{1800}$	$\frac{1}{2400}$	$\frac{1}{3600}$
2	1605	Doubtful			++	++	++	++	++	++	+	+	−	−	−
3	1527	1	2	2	+	++	−	−	−	−	−	−	−		
4	1734	2	2	2	+	++	−	−	−	−	−	−	−		
8	1588	1	2	2	−	+	−	−	−	−	−	−	−		
9	1696	2	2	2	−	+	−	−	−	−	−	−	−		
17	1365	1	1	1	−	+	−	−	−	−	−	−	−		
18	1306	1	1	1	−	±	−	−	−	−	−	−	−		
21	1452	1	1	2	−	+	−	−	−	−	−	−	−		

The antigen was obtained from the Central Medical Department Laboratory, it having been made from a single strain of *B. mallei* previously isolated at Neufchateau. Two series of reactions were performed as shown by tables 1 and 2.

The agglutination reactions were carried out in dilutions varying from 1:400 to 1:3600 inclusive. The antigen used was obtained from Neufchateau. The strain possessed a characteristic morphology, giving as well a typical growth upon potato media. The tests were carried out as follows: a twenty-four to forty-eight-hour growth on glycerin agar slants was emulsified in physiological saline. Two series of reactions were performed, in the first the dilutions ranging from 1:400 to 1:900 inclusive, while in the second series the dilutions ran from 1:600 to 1:3600 inclusive. In the first series the suspension was employed unheated, while for the second series it was heated at 60°C. for one-half hour. A uniform light suspension was used in both series. After being set up the tubes were kept for one-half hour in a water bath at 56°C., then for twenty-four hours at 37°C., likewise in a water bath, and finally in an ice box over night, when final readings were made. The horse sera used were heated at 56°C. for one-half hour, while the mule sera were exposed to 62°C. for the same time. The controls were carried through with the same technic as was used for the first series. Being all horse sera, they were heated at 56°C. for one-half hour.

From tables 1 and 2 it will be seen that serological reactions were carried out on 94 horses and 8 mules. The mallein tests recorded were done upon February 9, 1919, being read by Majors McKillip, Ratigan, and Gould of the Veterinary Corps. They are recorded by figures 0, 1, or 2, it being the old system of reading these reactions, 1 being doubtful, and 2 suspicious. The complement fixation reactions were performed in duplicate, the same technic being used for both series. The agglutination reactions were likewise carried out in duplicate, the second series differing in that the emulsion was inactivated for one-half hour in a water bath at 60°C., as previously explained. Readings: a double plus means complete fixation or complete agglutination, while one plus expresses almost complete fixation or agglutination;

a plus-minus means slight fixation or agglutination, while a dash represents complete hemolysis or no agglutination.

Complement fixation reactions. Horses: From a study of table 1, it will be seen that 71 of the 94 sera from horses gave a positive complement fixation. Twenty-three of the sera were negative.

TABULATED: HORSES 94	NUMBER	PER CENT
Complement fixation (++) twice	13	13.8
Complement fixation (++) once, (+) or (±) once	6	6.3
Complement fixation (++) at least once	19	20.1
Complement fixation (+) twice	33	35.2
Complement fixation (−) or (±)	23	24.4
Complement fixation (++) or (+) at least once	71	75.4

It was impossible to control these complement fixation reactions with absolutely known positive and negative sera, but the fact that in both series there were reactions where there was complete fixation and no fixation would indicate that they controlled themselves. Throughout both series, two sera, serial nos. 20 and 91, were the only ones found to be anticomplementary and these were both in the second series done on February 17, 1919.

Agglutination reactions. Horses. In consideration of the frequent presence of agglutinins even in a dilution of 1:400 to 1:500 in normal horse sera, and the fact that the sera were heated to 56°C. for one-half hour, we have adopted as a dividing line between a positive and a negative result a dilution of 1:600. Reactions to the degree of double plus or plus with a dilution of 1:600 have been considered as positive.

TABULATED: HORSES 94	NUMBER	PER CENT
Positive	42	44.7
Borderline	12	12.7
Negative	40	42.5

Borderline reactions are those which gave a single plus in one series at a dilution of 1:600 while failing to give a plus or more in the same dilution in the other series. Considering the borderline cases in conjunction with the definitely positive ones a percentage of 57.4 is obtained.

Complement fixation. Mules. The sera from eight mules giving mallein reactions of the same type were carried through the same tests. Certain difficulties were experienced with the mule sera, they being partly due to their being inactivated at 62°C. for one-half hour while the horse sera were inactivated at 56°C. for the same time. One of these sera coagulated at this temperature; animal no. 1391, necessitating its being discarded from the series. Others showed certain physiochemical alterations resulting in the sera becoming of a viscid consistency, although still remaining liquid. The discrepancies between the two complement fixation reactions we cannot explain. One serum, serial no. 2, was anticomplimentary on the second test. Another interesting fact is that due to an oversight, the serum of mule, serial no. 2 was inactivated at 37°C. instead of at 62°C. Both fixation reactions in this case resulted in a (++) fixation, although the second test was anticomplementary. Further this was the only mule serum that showed any agglutination, in this case being positive up to a dilution of 1:1200.

Agglutination reactions. Mules. In our opinion the agglutination reactions as carried out upon these eight mule sera are subject to severe criticisms. It has been stated previously that they were heated at 62°C. for one-half hour. We think that the heating of the sera to this temperature for that time would destroy almost all, if not completely, the agglutinins present. This opinion is rather corroborated by the agglutination reactions in the case of mule, serial no. 2, where the serum was heated to only 37°C. for one-half hour.

Complement fixation and agglutination. Taking these two reactions in conjunction we find that 38 times out of the 94 horses both reactions were positive in the same animal. This gives a percentage of 40.4. In 15 instances both reactions were negative in the same animal giving a percentage of 15.9.

Controls. As a control upon the above work, complement fixation and agglutination reactions were carried out upon 51 horses which gave a negative intra-palpebral mallein test. There were no mule controls. The complement fixation reactions were carried through with the same technic as used for the horse series. The agglutination reactions were accomplished by the same methods as were used in the first horse series. Out of the 51 tests, 3 gave a one plus reaction, while 2 others gave a plus-minus result. In no case was the serum anticomplementary. These results are satisfactory in that they indicate that there would be an error in only 5.8 per cent of the cases.

Agglutination. The controls are very satisfactory since in not a single case do they show a one plus reaction with a 1:600 dilution. Considering the agglutinins that are often present in normal horse sera, this series would tend to indicate a very low average agglutination titer among the normal horses. Also it would indicate that our choice of a 1:600 titer, reading one plus, was well inside the safe limit as a diagnostic line, which would indicate that the borderline cases in the suspicious horse series were probably positive cases.

SUMMARY

There are certain factors that should be taken into consideration in interpreting these serological results. The fact that the sera of all the suspicious horses and controls were heated at 56°C. for one-half hour would tend to cut down the agglutinating titer of those sera. However, as the same technic was carried out in both test and control animals the results have a true relative significance. This fact may account for the absence of agglutinins in such a large percentage of the normal control horses.

The results definitely indicate that in horses giving a suspicious intra-palpebral mallein reaction of this type the complement fixation test is of more value than the agglutination reaction. In the former 75.4 per cent were positive while in the latter only 57.4 per cent were positive, even when all the borderline cases were included.

These observations agree fairly well with published statistics. In Povitzky's recent article (this Journal, 1918, **3**, 463) she records work done in the laboratories of the New York City Department of Health on 123 horses which were proven at autopsy to have glanders. The complement fixation reaction was positive in 75.6 per cent of these cases, while the agglutination reaction was positive in 64.2 per cent. In her work she used horse sera which had not been heated to 56°C. for one-half hour, but again this would be partly neutralized by her using a titer of 1:1000 as necessary for a positive result. The consensus of opinion is that the agglutination reaction gives higher positive results in acute cases, while in chronic or subacute cases the complement fixation is the more reliable.

CONCLUSIONS

In consideration of the problem in view, that is, whether doubtful or suspicious intra-palpebral mallein reactions in horses are confirmed by the serological findings, our facts would indicate that the complement fixation reaction is of greatest value, it being positive in 75 per cent of the 94 horses examined. The agglutination reaction ranks second, confirming a suspicious mallein test with a definitely positive reaction in 44 per cent of the cases in the same series. Therefore we would conclude that the complement fixation reaction is of the greatest benefit in confirming a doubtful intra-palpebral mallein test, but that this reaction should be considered in conjunction with an agglutination test, one to act as a check upon the other.

COMPARISON OF SMEAR, CULTURE AND COMPLEMENT FIXATION IN CHRONIC GONORRHOEA IN WOMEN

A PRELIMINARY REPORT

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CLINICAL

The clinical material, serums and film preparations on which this report is based, were obtained from patients under treatment on the Venereal Disease Service of the Department of Health Hospitals of New York City. Ninety per cent of these patients are public prostitutes, the remainder being of the clandestine type. Their ages range from sixteen to sixty, the average being in the early twenties.

For convenience in outlining treatment and presenting the necessary data for the bacteriologist, the cases on admission are divided into three general classes, acute, subacute and chronic.

Acute

Vulva. Active inflammation and swelling of the parts. Involvement of Bartholin glands with or without abscess formation, but exhibiting some evidence of inflammation in the duct.

Urethra. Evidence of active inflammation as witnessed by mucopurulent or purulent discharge and swelling of the mucous membrane at the mouth of the urethra and ability to express a drop of purulent material on massaging Skenes glands.

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[1] Clinical study.

[2] Laboratory studies.

Vagina. Inflammation present in varying degrees; mucous membrane swollen, reddened and sensitive.

Cervix uteri. Presents a swollen and reddened appearance with or without erosions and a muco-purulent discharge.

Subacute

Those cases presenting any or all of the symptoms of the acute stage but in less intensified form and showing usually erosions of the cervix. The discharge is free and mucopurulent in character.

Chronic

Those cases presenting no evidence of active inflammation and exhibiting some or all of the following symptoms in varying degrees.

Discharge. Slight or profuse; mucopurulent in character.

Bartholin glands. Evidence of involvement as witnessed by fibrous change in the gland itself, with or without patent duct, and showing purulent discharge.

Urethra. No inflammatory condition apparent. On massage pus obtained from Skenes glands.

Cervix. May or may not be enlarged. Canal usually unduly patent. Erosions fairly constant. Mucopurulent discharge. Evidence of prior adnexa involvement such as thickened tubes, loss of mobility of uterus; evidence, or history, of previous pelvic or abdominal surgery.

In table 1 are given a few of our cases which are representative of all. If plotted these would show a remarkable similarity to the Wassermann curve in a luetic responding to treatment. In the cases shown, the complement deviation has at no time been at variance with the clinical picture.

In the acute and early subacute cases, we have had positive cultures and smears when the complement fixation reaction was negative. In each instance there was no doubt of the infection being a recent one in very young girls from sixteen to twenty years of age and of the clandestine type of prostitute.

In the chronic cases, our experience has shown the complement fixation test to give as high a percentage of positives as does the Wassermann reaction in old luetics.

TABLE 1

Showing the gradual reduction of the complement fixation. Bleedings were made at seven day intervals

PATIENT	GONOCOCCUS COMPLEMENT FIXATION REACTIONS									
1	++++	++++	++	++	++	++	++	+	±	±	
2	++	+	+	+	+	±	±	±	±	±	
3	++	+	+	+	±						
4	±	±	±	±	±	±	±	±	−	−	−
5	++++	++	++	+	+	+	±				
6	++++	++++	+++	+++	++						
7	+++	+++	+	+	±	±	±	±	−	−	
8	++++	+++	+++	+++	+++	+	+	+	±	−	−
9	++++	+++	+++	+	+	±					
10	±	±	±	±	−						

LABORATORY STUDIES

Our laboratory studies have been concentrated upon experiments to improve the methods for diagnosis by smear, culture and complement fixation. A trial of many methods for each of those forms of diagnosis led us to adopt the following technic as our standard for routine examinations.

Technic and diagnosis

Smear. The smears were made by the clinician at the time the cultures were made. A drop of the discharge material was taken with a sterile platinum loop and smeared upon the surface of a sterile slide. When very little material was obtainable, it was found to be an advantage to place a drop of sterile water on the slide and gently manipulate the loop containing scant secretion in that. By this means the leucocytes were not destroyed and we succeeded in making a good smear in many instances where we had failed to do so without the water. After drying and fixing, the smear was stained by Gram's method. During the first months of our studies when stable staining powders were available, we used the Nicolle modification of the Gram method. Later we used the Leitz aniline gentian violet powder with Stirling's method. Before staining the smears, we tested all of the staining solutions with twenty-four-hour cultures of *Staphylococcus pyogenes aureus* and *Bacillus coli.* Without these control tests, we consider the Gram method to have no value as a differential stain.

Smear diagnosis. We have followed the Williams (1) rules for smear diagnosis:

1. Positive smears. Those showing leucocytes filled with morphologically typical gonococci.

2. Suspicious smears. Those showing any suspicious intracellular diplococci and 50 per cent, or more, of polymorphonuclear leucocytes.

3. Observation smears. Those showing 50 per cent, or more, of polymorphonuclear leucocytes, but no suspicious intracellular diplococci; or, those having the clinical symptoms of discharge and inflammation and showing less than 50 per cent of polymorphonuclear leucocytes.

4. Negative smears. Those showing less than 50 per cent of polymorphonuclear leucocytes, no suspicious intracellular diplococci and no clinical evidence of the disease.

Culture. Plates of glycerine-veal-horse-serum-agar, streaked with rabbit, human, or horse blood, were inoculated by means of a platinum loop. The discharge material was streaked across the surface of the plate. The plates were incubated at 36°C. for one week and examined daily. The average optimum growth of the gonococcus in the isolation culture is forty-eight hours. In two of the cases, the gonococcus was isolated from a three day plate that had shown no growth within forty-eight hours. Occasionally the colonies develop within twenty-four hours. All colonies appearing suspicious were fished under the microscope with a magnification of three hundred diameters. The pure cultures of gonococci were grown upon glycerine-veal-horse-serum-agar for several generations until they could be grown upon a serum-free medium. Some of the gonococci isolated in this study made a fair growth upon disodium phosphate agar in the fourth generation, but most of the cultures had to be kept on a serum medium a longer time. This is necessary before transplanting to the North medium, but once induced to grow upon it they have continued to grow well. None of the gonococcus cultures have grown upon ordinary agar.

Culture diagnosis. We have diagnosed as *positive* those cultures from which we have isolated in pure culture a biscuit-shaped Gram-negative diplococcus, dividing at right angles, that does not grow in early generations without the aid of blood-serum. This rule for diagnosis excludes the *Micrococcus catarrhalis* and other Gram-negative cocci that grow immediately upon ordinary agar. At this point, it may be well to emphasize the importance of obtaining a growth from the fishing transplant before making a diagnosis of the culture. We consider a diagnosis unreliable which is made upon the macroscopic or low-power microscopic appearance of a colony, unless it is corroborated by a fishing showing typical gonococci in smear. This is essential for two reasons: first, that several other organisms make similar colonies upon this medium; and, second, that the gonococcus does not always make the typical colony.

We have diagnosed as *suspicious* all cultures in which we have found colonies showing suspicious Gram-negative diplococci in a smear of the colony and from which we have obtained no growth after fishing; we have considered suspicious also, all cultures from which we have obtained mixed subcultures containing typical or suspicious Gram-negative diplococci which have not been isolated in pure culture. In some instances the Gram-negative cocci have not survived the repeated transplants necessary to free them from the associated growth of the other organisms and, therefore, cannot be definitely classed.

We have diagnosed as *negative* all cultures from which we have failed to obtain typical or suspicious colonies that have given in fishings suspicious Gram-negative cocci. A large number of suspicious colonies having marked central granulation, have given in fishings pure cultures of a minute Gram-negative bacillus and also pure cultures of a lancet-shaped Gram-positive coccus.

Complement fixation. Antigen. The complement fixation tests reported here have all been made with an antigen prepared from the strains of gonococci isolated by Torrey several years ago. The gonococci were grown upon disodium phosphate agar for forty-eight hours at 37°C. They were then treated with alcohol and ether to remove the lipoids; dried, weighed, powdered and suspended in saline in the proportion of 1 gram of the dried powder to 200 cc. of physiological saline solution. This suspension was heated in the water-bath at 80°C. for one hour and was then ready for standardization. The standard dose of our antigen contains two fixation units and one-fourth, or less, of the anticomplementary unit. This antigen is stable when kept at ordinary ice-box temperature. Freezing does not injure it. No preservative is used.

Serum. The patient's serum was inactivated before the test and was used undiluted.

Complement. Guinea-pig complement was used. The serums from all guinea-pigs were tested for natural antisheep amboceptor, for hemolytic activity, for anticomplementary reaction with the antigen and the control serum and for fixability with the antigen and the control serum, before being pooled for tests. These preliminary tests are essential for the reason that some guinea-pig serums are not fixable by gonococcus antigen and serum; if non-fixable serums are included in the pooled complement, false negative reactions of the patient's serum may be obtained.

Hemolytic system. The antisheep system was used. The dose of complement contained two hemolytic units. The hemolytic unit was obtained by titrating a 10 per cent dilution of the pooled complement with sensitized cells. The sensitized cell dose contained two hemolytic units of amboceptor and 0.1 cc. of a 5 per cent suspension of sheep-cells. The complement unit was read at the end of thirty minutes in the water-bath at 37°C.

The test. One-tenth of the original Wassermann volumes of all reagents was used. The tests were made in duplicate with controls for hemolytic system, for anticomplementary reaction of the patient's serum, for natural antisheep amboceptor in the patient's serum and for fixation unit and anticomplementary reaction of the antigen.

Complement fixation diagnosis. The Citron (2) method for diagnosis was followed.

TABLE 2

Showing the incidence of the complement fixation reaction in comparison with smear and culture findings

CLINICAL DIAGNOSIS	NUMBER OF CASES	SMEAR DIAGNOSIS	CULTURE DIAGNOSIS	COMPLEMENT FIXATION DIAGNOSIS				
				Strong positive	Positive	Weak positive	Doubtful	Negative
Mild chronic gonorrhoea	21	Neg.	Neg.	2	5	9	4	1
	22	Ob.*	Neg.	5	7	7	2	1
	3	Pos.	Pos.	0	2	0	1	0
	4	Ob.	Pos.	1	1	2	0	0
Totals	50			8	15	18	7	2
Controls: Clinically negative								
Children	8	...	...	0	0	0	0	8
Adults	50	...	...	0	0	0	0	50

Totals in percentages: 82 per cent gave some degree of positive fixation; 14 per cent gave doubtful fixation; 4 per cent gave negative reaction.

* Observation.

We have charted fifty cases that were diagnosed as clinically mild chronic gonorrhoea and fifty-eight control cases that were considered to be clinically negative. The chronic gonorrhoeal cases were examined by smear, culture and complement fixation. The control cases had the complement fixation test only. The incidence of complement fixation with the gonococcus antigen compares closely to that reported by McNeil (3) (4). We have not been able to group these reactions in relation to the duration of the disease because we have not been able to obtain such data from the patients.

CONCLUSIONS

The gonococcus complement fixation test is of undoubted value in chronic gonorrhoeal infections.

In acute and early subacute infections it is on a par with the Wassermann test in the initial lesion stage prior to development of the secondary.

A non-gonorrhoeic does not give a positive complement fixation test. (Table 2.)

A gonorrhoeic may give a negative test in certain stages of the disease.

REFERENCES

(1) WILLIAMS: Collected Studies. Res. Lab. N. Y. H. D., 1911, **6**, 29.
(2) CITRON: Immunity. Translated by Garbat, ed. 1914, 184.
(3) SCHWARTZ AND McNEIL: Amer. Jour. Med. Sci., 1911, **141**, 693.
(4) McNEIL: N. Y. Med. Jour., 1914, **100**, 350.

EXPERIMENTS UPON THE PRODUCTION OF ANTIHUMAN HEMOLYSIN WITH SPECIAL REFERENCE TO IMMUNIZATION WITH ERYTHROCYTES SENSITIZED WITH HEATED SERUM

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Undoubtedly the usual difficulties encountered in the preparation of highly potent antihuman hemolytic sera by the immunization of rabbits with human erythrocytes, have checked the wider adoption of an antihuman hemolytic system for complement-fixation tests. It would appear to be the common experience of most serologists that rabbits withstand immunization with human erythrocytes rather poorly, many animals losing rapidly in weight and succumbing before their sera are sufficiently hemolytic for practical purposes, either suddenly with symptoms of anaphylaxis or more slowly with general wasting.

Three causes have been generally assigned for these results, namely: (1) that animals succumb to anaphylactic shock; (2) that fatal embolism may occur due to agglutination in vivo of the injected cells and (3) that human erythrocytes are highly toxic for rabbits. Various methods have been proposed from time to time for the preparation of antihuman hemolysin and in a recent study of these by Kolmer and Rule (1), the daily intravenous injection of 0.1 cc. washed human erythrocytes was found to give best results, the mortality being lower and hemolysin production good after twenty-one or more injections.

Kolmer and Rule have assigned toxicity of human erythrocytes for rabbits, and probably agglutination in vivo, as being the more prominent causes of deaths during immunization rather than anaphylaxis, which is generally held responsible. An experiment by Vedder (2) tends to show that agglutination in vivo may play an important rôle; Vedder heated the diluted serum of an immunized rabbit at 70° to 80°C. for one hour to destroy agglutinins and produce agglutinoids and found that washed cells exposed to this heated serum were rendered insusceptible to agglutination, and probably better borne when injected intravenously into a highly sensitized rabbit than untreated cells.

At the suggestion of Professor Kolmer I have repeated Vedder's experiment and have also made comparative studies on the production of antihuman hemolysins and hemagglutinins in rabbits by the intravenous injection of unwashed cells carrying serum, thoroughly washed cells free of serum and washed cells rendered unsusceptible to agglutination either totally or partially by exposure to heated immune serum, to determine more definitely the rôles of anaphylaxis and agglutination in vivo as the probable causes of fatalities among rabbits during immunization with human cells.

TECHNIC

Human corpuscles were secured by defibrination and centrifuging; to one series of rabbits 0.5 cc. of these unwashed and packed cells suspended in 5 cc. of saline solution were injected intravenously every five days.

A second series of animals received similar injections after the corpuscles had been *washed* with large volumes of saline solution at least five times, the last fluid yielding negative reactions for proteins.

A third series received similar injections of corpuscles washed three times, then exposed for one to one and one-half hours to 10 cc. of antihuman sera which had been diluted 1:10 and heated at 70° to 80°C. for one hour, followed by two additional washings to remove serum.

Four days after each injection each animal was weighed and a small amount of blood removed from an ear vein; the sera were heated at 56°C. for thirty minutes and tested for agglutinins and hemolysins as follows:

Agglutination tests: 1 cc. of varying dilutions of serum were added to 1 cc. of 1 per cent suspensions of mixed washed corpuscles from several persons, incubated in a water bath at 38°C. for one hour and read macroscopically after standing in a refrigerator over night.

Hemolysin tests: 1 cc. of varying dilutions of serum were mixed with 0.9 cc. of 1 per cent suspension of mixed washed corpuscles of several persons and 0.1 cc. of 40 per cent dilution of mixed guinea-pig complement sera; these were incubated in a water bath for one hour and the results read after standing in a refrigerator over night.

RESULTS

When 0.5 cc. of washed packed human corpuscles are mixed with 10 cc. of 1:10 dilution of immune antihuman serum heated at 70° to 80°C. for one and one-half hours and left standing at room temperature for one hour, the corpuscles are usually rendered less susceptible to agglutination by unheated immune serum. This is shown in table 1, giving a summary of the results of experiments with five immune sera; presumably the decrease in susceptibility of the heated corpuscles to agglutinins is due to the union of agglutinoids to the corpuscles, as explained by Vedder.

TABLE 1

The agglutinability of human erythrocytes after exposure to various antihuman sera diluted 1:10 and heated at 70° to 80°C. for one and one-half hours

CORPUSCLES	RESULTS WITH IMMUNE SERA FROM				
	Rabbit 1	Rabbit 2	Rabbit 3	Rabbit 5	Rabbit 6
Plain	1:800	1:800	1:600	1:1600	1:2400
Sensitized (exposed)	1:300	1:100*	1:100*	1:100*	1:100*

* Less than this.

These exposed corpuscles are, however, more susceptible to hemolysis, as shown in table 2, summarizing experiments with nine immune sera. This is due to the fact that immune hemolysins are probably more resistant to heat than the agglutinins and that the corpuscles were actually sensitized with unchanged hemolytic amboceptors rather than with amboceptoids.

Table 3 gives a summary of the results of agglutination tests with the sera of rabbits tested four days after each of nine injections of unwashed, washed and sensitized (to heated serum) corpuscles; table 4 gives a summary of the hemolytic titers of the sera.

Charts 1 and 2 show in a graphic manner the *average* agglutinating and hemolytic titers of the animals immunized with the unwashed, washed and sensitized corpuscles.

Table 5 shows the hemolytic titers of the sera of those rabbits surviving nine injections of the unwashed, washed and sensitized corpuscles; each serum was diluted 1:40 after heating at 56°C. for thirty minutes and tested in amounts of 0.1 to 0.5 cc. with 1 cc. of 1 per cent suspension of corpuscles and 0.1 cc. of 40 per cent of guinea pig complement.

TABLE 2

The susceptibility to hemolysis of human erythrocytes after exposure to various anti-human sera diluted 1:10 and heated at 70° to 80°C. for one and one-half hours

CORPUSCLES	HEMOLYTIC ACTIVITIES OF SERA FROM						
	Rabbit 2	Rabbit 3	Rabbit 5	Rabbit 6	Rabbit 7	Rabbit 9	Rabbit 10
Plain	1:100	1:200	1:100	1:200	1:200	1:100*	1:100
Sensitized (exposed)	1:800	1:200	1:800	1:800	1:800	1:800	1:800

* Less than this.

Table 6 shows the weights of the animals during the period of immunization as an index of the influence of immunization upon the general condition of rabbits receiving injections of unwashed, washed and sensitized corpuscles.

These results may be summarized as follows:

1. Rabbits receiving intravenous injections of 0.5 cc. of packed unwashed, washed and sensitized corpuscles (exposed to immune serum heated at 70° to 80°C.) every five days showed no differences in body weight and the death rate was the same.

TABLE 3

The production of immune antihuman hemagglutinins

BY	AGGLUTINATION TITERS FOUR DAYS AFTER								
	First injection	Second injection	Third injection	Fourth injection	Fifth injection	Sixth injection	Seventh injection	Eighth injection	Ninth injection
Unwashed corpuscles	1:8	1:80	1:800	1:2000	D.†				
	1:32	1:256	1:800	1:4000	1:2000	1:8000	1:2000	1:2000	1:3000
	1:16	1:128	1:800	1:4000	1:4000	1:4000	1:2000	1:4000	1:5200
Washed corpuscles	1:32	1:200	1:1600	1:8000	1:4000	1:4000	1:2000	D.†	
	1:32	1:256	1:6400	1:8000	1:8000	1:8000	1:8000	1:4000	1:3000
	1:32	1:256	1:800	1:4000	1:2000	1:8000	1:4000	1:4000	1:4000
Sensitized corpuscles*	1:8	1:128	1:3200	1:4000	1:4000	1:4000	1:4000	1:4000	1:4600
	1:4	1:64	1:800	D.†					
	1:64	1:128	1:400	1:4000	1:4000	1:4000	1:2000	1:4000	1:4000
	1:64	1:256	1:3200	1:4000	1:4000	1:4000	1:4000	1:4000	1:3400

* Corpuscles exposed to immune serum diluted 1:10 and heated at 70° to 80°C. for one and one-half hours and then thoroughly washed.

† Died.

TABLE 4

The production of antihuman hemolysin

IMMUNIZATION	HIGHEST DILUTION IN 1 CC. PRODUCING COMPLETE HEMOLYSIS								
	After first	After second	After third	After fourth	After fifth	After sixth	After seventh	After eighth	After ninth
Unwashed corpuscles	0*	0	0	1:10	Died				
	0	0	0	1:20	1:50	1:50	1:50	1:50	1:100
	0	0	0	1:40	—	1:50	—	1:50	1:100
Washed corpuscles	0	0	1:20	1:80	—	1:50	1:50	Died	
	0	0	1:20	1:40	—	1:50	1:50	1:50	1:200
	0	1:8	1:40	1:80	1:50	1:50	1:50	1:100	1:200
Sensitized corpuscles	0	0	1:10	—	1:50	1:50	1:50	1:50	1:200
	0	0	0	Died					
	0	0	0	1:10	1:50	1:50	1:50	1:50	1:50
	0	0	1:40	1:80	—	1:50	1:50	1:50	1:100

* Hemolysis absent or incomplete in 1 cc. of 1:10 dilution.

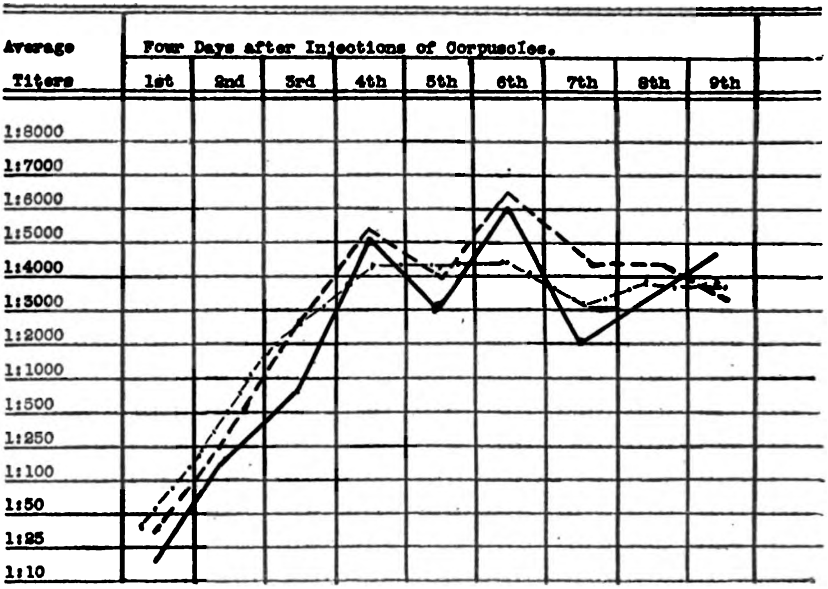

CHART 1. PRODUCTION OF IMMUNE ANTIHUMAN HEMAGGLUTININS

——————— By intravenous injection of rabbits with unwashed corpuscles.
- - - - - - - - By intravenous injection of rabbits with washed corpuscles.
-·-·-·-·-·- By intravenous injection of rabbits with corpuscles, exposed to immune serum 1:10 heated at 70° to 80°C. for one and a half hours.

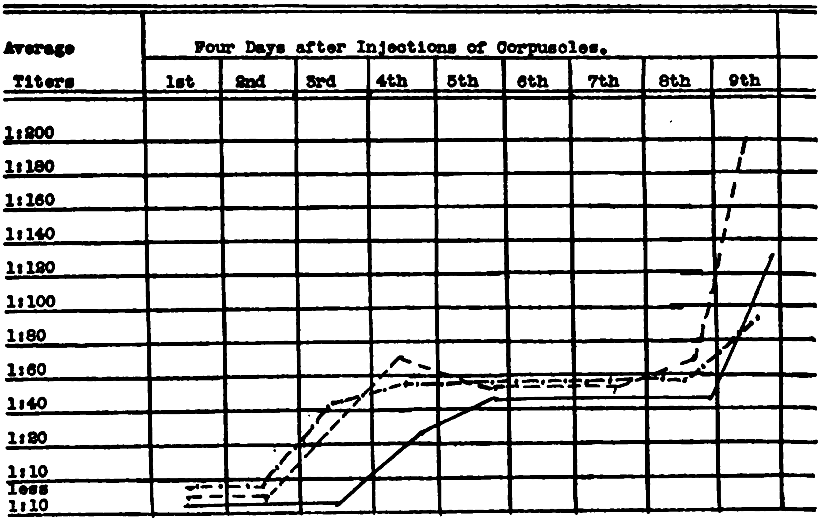

CHART 2. PRODUCTION OF IMMUNE ANTIHUMAN HEMOLYSINS

——————— By intravenous injection of rabbits with unwashed corpuscles.
- - - - - - - - By intravenous injection of rabbits with washed corpuscles.
-·-·-·-·-·- By intravenous injection of rabbits with corpuscles exposed to immune serum 1:10 heated at 70° to 80°C. for one and a half hours.

TABLE 5

Final hemolytic activity of heated immune sera after nine injections of human erythrocytes

IMMUNIZATION	DOSES OF HEATED SERUM DILUTED 1:40				
	0.1 cc.	0.2 cc.	0.3 cc.	0.4 cc.	0.5 cc.
Unwashed corpuscles	M*	M	M	C	C
	M	M	C	C	C
Washed corpuscles	M	C	C	C	C
	M	M	C	C	C
Sensitized corpuscles	M	M	M	C	C
	M	M	M	M	M
	M	M	M	C	C

* M = marked hemolysis; C = complete hemolysis.

TABLE 6

The influence upon body weight of rabbits receiving intravenous injections of human erythrocytes

IMMUNIZATION	BODY WEIGHTS IN GRAMS FOUR DAYS AFTER INJECTIONS								
	Before	First	Second	Third	Fourth	Fifth	Sixth	Seventh	Eighth
Unwashed corpuscles	1750	1700	1800	1500	1300	Died	—	—	—
	1700	1800	1900	1800	1900	2000	2100	2200	2100
	1650	1500	1700	1600	1600	1700	1400	1500	1350
Washed corpuscles	1700	1600	1600	1600	1500	1600	1600	1880	Died
	1700	1400	1500	1500	1400	1400	1300	1500	1500
	2250	2200	2200	2000	2100	2000	2000	2150	1700
Sensitized corpuscles	2400	2100	2000	1700	1800	1700	1700	1850	1800
	2300	2400	2200	2100	Died	—	—	—	—
	2250	2400	2500	2100	2300	2300	2500	2560	2400
	2700	2100	2000	1800	2100	2000	2000	2300	2100

2. Under these conditions, apparently, unwashed corpuscles carrying human serum were not any more likely to kill rabbits than washed cells, which tends to minimize the importance attached to anaphylaxis as a cause of death among immunized animals when the injections are closely spaced.

3. Apparently corpuscles exposed to heated immune sera and thereby rendered less susceptible to agglutination but more

susceptible to hemolysis, are just as toxic for rabbits as plain cells; this tends to minimize the importance attached to agglutination in vivo as a cause of death of rabbits during immunization with human corpuscles but does not exclude the possibility of fatal agglutination in vivo inasmuch as the corpuscles were not totally insusceptible to agglutination.

4. The agglutinin and hemolysin production by unwashed, washed and sensitized corpuscles were practically the same, allowing for marked individual variations among animals.

When rabbits were immunized with human corpuscles injected intravenously and intraperitoneally at intervals of seven days or less, the gradual loss in weight and high mortality are apparently due primarily to the toxicity of human corpuscles for these animals; this appears particularly true of animals succumbing one or more days after an injection. Sudden fatalities with convulsions are probably most frequently caused by agglutination and hemolysis in vivo rather than by true anaphylactic shock.

Additional experiments have shown that the stromata of human corpuscles when injected intravenously into fresh rabbits (not previously injected) may produce immediate death and presumably by agglutination in vivo and embolism. Vedder had described a method of immunization with "shadow corpuscles," hemolyzed after preliminary treatment with illuminating gas, which he believes superior to the usual method of injecting whole cells. Kolmer and Rule found this method fairly satisfactory for the production of hemolysins but in a series of comparative experiments the mortality rate was practically the same as that accompanying immunization with whole cells.

Solutions of human erythrocytes prepared by dissolving each cubic centimeter of washed packed corpuscles in 5 cc. of sterile distilled water for two hours at room temperature followed by thorough centrifuging and rendering the fluid isotonic with sodium chlorid, have proven somewhat less toxic for rabbits upon intravenous injection than suspensions of whole cells. It is also noteworthy that the injection of these solutions results in the production of much less agglutinin than whole cells (table 7) and likewise somewhat less hemolysin (table 8); these results are similar to those reported by Kolmer and Rule (1) and they support the findings of Bordet (3) and Levene (4) indicating that hemoglobin stimulates the production of hemolysins rather poorly.

According to these results it would appear that the stromata of human corpuscles are more dangerous for the rabbit either by agglutination in vivo or by direct toxicity than solutions largely free of stromata; accordingly, immunization with watery solutions of human erythrocytes largely free of cellular fragments may prove the method of choice, although more prolonged immunization may be required for the production of sera of sufficient hemolytic activity for complement fixation tests.

TABLE 7

The production of immune antihuman hemagglutinins

ANTIGENS	TITERS AFTER INJECTIONS				
	Second	Third	Fourth	Fifth	Sixth
Whole corpuscles	1:2	1:40	1:640	1:700	1:460
Hemolyzed corpuscles	1:2*	1:2*	1:64	1:45	1:40

* Less than this.

TABLE 8

The production of immune antihuman hemolysins

ANTIGENS	TITERS AFTER INJECTIONS				
	Second	Third	Fourth	Fifth	Sixth
Whole corpuscles	1:2*	1:30	1:30	1:45	1:24
Hemolyzed corpuscles	1:2*	1:5	1:15	1:13	1:18

* Less than this.

SUMMARY

1. Immunization of rabbits with unwashed and washed human erythrocytes by intravenous injection every five days, produced similar results in influence upon body weight, mortality, hemagglutinin and hemolysin production.

2. Exposure of human erythrocytes to antihuman serum diluted 1:10 and heated at 70° to 80°C., reduces susceptibility

to agglutination and increases susceptibility to hemolysis; immunization of rabbits with these sensitized erythrocytes produced results similar to those observed with plain washed corpuscles.

3. Stromata of human erythrocytes injected intravenously into rabbits may prove fatal; solutions of erythrocytes in distilled water largely free of stromata (shadow corpuscles) were better borne.

CONCLUSIONS

1. The high mortality among rabbits immunized with human erythrocytes is probably due primarily to direct toxicity of the cells and agglutination and hemolysis in vivo, rather than anaphylaxis.
2. The stromata of human corpuscles (shadow corpuscles) may prove fatal for rabbits when injected intravenously, probably by agglutination in vivo.
3. Solutions of human erythrocytes in distilled water largely free of shadow corpuscles are much less toxic and better borne by rabbits than suspensions of whole cells; these solutions produce much less hemagglutinin and slightly less hemolysin than whole cells, but may prove preferable to the latter for the immunization of rabbits for the preparation of hemolysin.

REFERENCES

(1) **Kolmer**, J. A., and **Rule**, A.: A study of methods for the preparation and preservation of hemolysins. Amer. Jour. Syphilis, 1920, July.

(2) **Vedder**, E. B.: The Production of Anti-human Hemolysin. Jour. Immunology, 1919, **3**, 141-146.

(3) **Bordet**, J.: Sur l'agglutination et la dissolution des globules rouges par le sérum d'animaux injectes de sang défibrine. Ann. Inst. Pasteur, 1898, **12**, 688.

(4) **Levene**, P. A.: On the production of hemolytic serum by injecting animals with different constituents of erythrocytes. Jour. Med. Research, 1904, **12**, 191-194.

A NOTE ON THE NON-SPECIFIC PRODUCTION OF ANTIBODIES

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During the course of experiments upon the production of immune agglutinins and hemolysins by the immunization of rabbits with sheep and human erythrocytes,[1] the sera where examined for the presence of agglutinins and hemolysins for sheep and guinea-pig corpuscles and for agglutinins for *B. typhosus* as well, as bearing upon the specificity of antibody production. Several investigators have reported experiments tending to show the non-specific production of such antibodies as agglutinins and hemolysins for sheep corpuscles during immunization of rabbits with human corpuscles; unfortunately these experiments were not always carefully controlled by a preliminary study of the sera of rabbits for natural antibodies and the selection of animals whose sera were free or almost so of these substances in order to avoid falling into the error of interpretating the presence of these natural antibodies as due to stimulation of body cells by a specific antigen.

In these experiments macroscopical agglutination tests were conducted with 1 cc. of varying dilutions of sera heated at 56°C. for thirty minutes, and 1 cc. of 2 per cent suspensions of corpuscles of persons, sheep and guinea-pigs; in addition forty-eight hour broth cultures of *B. typhosus* were employed. These mixtures were incubated at 38°C. in a water bath for one hour and the readings made after the tubes had stood in a refrigerator over night.

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[1] Matsumoto, M. Experiments upon the production of antihuman hemolysin with special reference to immunization with erythrocytes sensitized with heated serum. Jour. Immunol. (this number).

The hemolysin tests were conducted with 1 cc. of varying dilutions of unheated sera, 0.1 cc. of 40 per cent guinea-pig complement and 0.9 cc. of 2 per cent suspensions of corpuscles. These mixtures were incubated for one hour and the results read the following day.

The tables give the averaged highest dilutions of sera producing well marked or complete agglutination or complete hemolysis.

Rabbits were immunized with intravenous injection of washed human and sheep corpuscles every five to seven days; the sera were tested four days after each injection for agglutinins for human, sheep and guinea pig corpuscles and *B. typhosus* and for hemolysins for sheep, human and guinea pig corpuscles.

The results are given as averages in tables 1, 2, 3, and 4.

TABLE 1

The influence of injections of human corpuscles in rabbits upon agglutinins for human, sheep and guinea-pig corpuscles and B. typhosus

INJECTIONS	AGGLUTINATION TITERS FOR			
	Human corpuscles	Sheep corpuscles	Guinea-pig corpuscles	B. typhosus
Preliminary	—*	—	—	—
Second	1:4	—	—	—
Third	1:40	—	—	—
Fourth	1:640	—	—	—
Fifth	1:800	—	—	—
Sixth	1:480	—	—	—

* — = No agglutination or incomplete agglutination in 1:2.

TABLE 2

The influence of injections of human corpuscles in rabbits upon hemolysis for human, sheep and guinea-pig corpuscles

INJECTIONS	HEMOLYTIC TITERS FOR		
	Human corpuscles	Sheep corpuscles	Guinea-pig corpuscles
Preliminary	—*	1:2	1:2
Second	—	1:2	1:2
Third	1:30	1:4	1:2
Fourth	1:40	1:4	1:2
Fifth	1:50	1:4	1:2
Sixth	1:24	1:4	1:2

* — = No hemolysis or incomplete hemolysis in 1:2.

TABLE 3

The influence of injections of sheep corpuscles in rabbits upon agglutinins for sheep, human and guinea-pig corpuscles and B. typhosus

INJECTIONS	AGGLUTINATION TITERS FOR			
	Sheep corpuscles	Human corpuscles	Guinea-pig corpuscles	B. typhosus
Preliminary	—*	—	—	—
Second	1:3	—	—	1:2
Third	1:26	—	—	1:2
Fourth	1:40	—	—	—
Fifth	1:80	—	—	—
Sixth	1:480	—	—	—

* — = No agglutination or incomplete agglutination in 1:2.

TABLE 4

The influence of injections of sheep corpuscles in rabbits upon hemolysins for sheep, human and guinea-pig corpuscles

INJECTIONS	HEMOLYTIC TITERS FOR		
	Sheep corpuscles	Human corpuscles	Guinea-pig corpuscles
Preliminary	1:2	—*	1:2
Second	1:6	—	1:2
Third	1:40	—	1:4
Fourth	1:480	—	1:12

* — = No hemolysis or incomplete hemolysis in 1:2.

SUMMARY

1. The immunization of rabbits with washed human and sheep corpuscles results in the production only of specific agglutinins and hemolysins for human and sheep corpuscles respectively.
2. Occasionally rabbits showed an apparent slight increase of non-specific hemolysin or agglutinin, but these were so slight and irregular as to be explained by the normal variation of natural antibodies or experimental error.

THE SACHS-GEORGI PRECIPITATION TEST FOR SYPHILIS

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For several years investigators in various parts of the world have given more or less time and effort in attempts to find a test for syphilis less elaborate and complicated than the Wassermann test, and, if possible, more delicate and specific. These attempts have been partly successful, but with the exception of the Lange colloidal gold test on spinal fluid, none of the suggested procedures have been generally adopted.

One reason for the rather slow advance is the fact that we are uncertain as to the exact nature of the mechanism of the Wassermann reaction—whether it is caused by a specific antibody or by some other substance. The problem is still further complicated by the extremely difficult task of growing *Treponema pallidum* either on artificial media or in the lower animals. By close clinical studies of cases, however, and carefully correlated laboratory investigations much progress is being made.

In 1912, Klausner (1) reported his findings in the reaction between blood serum and distilled water. When these two are mixed together in the proper proportions a flocculent precipitate separates out, the amount of the precipitate being much greater in syphilitic than in normal sera.

The same year Lange (2) attempted to demonstrate a difference between normal and syphilitic sera by their relative precipitating properties for colloidal gold solutions. He was unsuccessful in this but discovered that spinal fluid in various dilutions gave most characteristic reactions.

About the same time the Porges-Hermann-Perutz reaction (3) was brought out in which equal parts of sodium glycocholate

(2 per cent solution) and an alcoholic cholesterol suspension (0.4 per cent) were used with inactivated patient's serum, a precipitate forming with syphilitic serum, while none forms with normal serum.

In 1914 Hirschfeld and Klinger (4) reported that tissue extracts digested with syphilitic serum lose their ability to coagulate blood.

In 1917 Vernes (5), in working on the precipitation of colloids, found that ferric hydroxide would precipitate the colloids of blood serum in certain dilutions, the dilutions being different in syphilitic sera from those in normal sera.

Of late considerable attention has been given to the Sachs-Georgi (6) test. This is essentially a precipitation caused by the mixture of syphilitic serum and cholesterinized antigen. In normal serum no precipitate occurs. The antigen (7) consists of an alcoholic extract of normal beef heart, to each 100 cc. of which is added 200 cc. of alcohol and 13.5 cc. of a 1 per cent solution of cholesterin. For use this is diluted one to five with physiological salt solution. In the test 0.5 cc. is mixed with 1 cc. of serum which has been diluted to 10 cc. with physiological salt solution. The results are read after twenty-four and forty-eight hours.

Because of the large volume in the Sachs-Georgi test, the rather scant precipitate often formed, and the length of time elapsing before the test is read, the present authors have devised a modification which has proven in their hands, much easier to manipulate and at the same time, no less sensitive in giving positive results with syphilitic sera. This modification differs from the original technic in that the antigen may be either cholesterinized or not; a much smaller volume is used—1.3 cc. instead of 10.5 cc.; and the tests may be read immediately, if centrifuged.

The antigen. The dilution of the antigen is of the greatest importance, careless addition of the salt solution often giving erroneous results. The required amount of undiluted antigen is placed in an Erlenmeyer flask and physiological sodium chloride added drop by drop with vigorous shaking until at least 5 cc. of the diluent has been run in. Larger quantities at a time can then be added but the vigorous shaking must be continued. The resulting suspension should be very turbid and milky; an opalescent suspension is an indication that the salt solution was added too rapidly without sufficient shaking. Such a suspension should be discarded as it will not give sufficiently sensitive results.

The antigen should be roughly titrated so that the optimum amount of precipitation will be obtained. To accomplish this, the antigen is diluted 1:10, 1:20, 1:40, 1:60, and 1:80, 1.0 cc. of each dilution being used with 0.3 cc. of a known positive serum. The dilution giving the optimum result should be used in the test.

Several different antigens have been used with little difference in results; three different lots of cholesterinized and two lots of plain alcoholic antigen. As a rule, the antigens giving the best results in the Wassermann test have served best here, and the dilution used for the Wassermann test has been proper in the precipitation test.

The serum. The prime requisite for the serum is that it must be clear. So far as age of the serum goes, no difference has been noted. Inactivation at 56°C. for thirty minutes apparently makes no difference in the results, therefore, in most of the work reported here, this procedure was omitted.

In the test, 0.3 cc. of serum is used, it having been found by trial to give optimum results. As low as 0.05 cc. gave a trace of precipitation and as high as 0.5 cc. has been used, but the amount indicated has been found best.

Time of reaction. The reaction between serum and antigen probably takes place instantaneously, though no change is apparent until after some hours of standing. If the tubes are whirled in the centrifuge a few moments, however, they may be read immediately. For tests in which the centrifuge is not employed, the mixtures are allowed to remain overnight when all the positive tests will show a precipitation. Occasionally a tube will require as long as forty-eight hours for the precipitate to come down, but after forty-eight hours no change has ever been noted.

Effect of temperature. Optimum results are obtained with low temperatures for incubation. If the tests are heated at 56°C. for thirty minutes immediately after mixing, no precipitation takes place on long standing either at room temperature or in the ice box. Thirty minutes at 37°C. does not retard later precipitation in the ice box, but no reaction has been obtained when the tests were allowed to remain at body temperature overnight. So far no appreciable difference has been noted in overnight incubation at room temperature and at ice box temperature, though the latter has usually been used.

THE PRECIPITATION TEST

In setting up the test, to each tube is added 0.3 cc. of clear serum and 1 cc. of antigen properly diluted. The tubes are shaken and either centrifuged and read immediately or allowed to stand overnight in the ice box.

TABLE 1

Comparison of Wassermann and precipitation tests on 296 sera

Both tests positive	65
Both tests negative	195
Positive precipitation, negative Wassermann	22
Negative precipitation, positive or doubtful Wassermann	14

Agreement with the Wassermann test has been obtained in a large percentage of cases. (Wassermann tests were run with two antigens; cholesterinized incubated at 37°C. for thirty minutes, and alcoholic incubated in ice box over night. The sheep cell system was used with previous extraction from the patient's serum of natural anti-sheep amboceptor.)

Out of 296 samples tested, results were obtained as shown in table 1.

It will thus be seen that 260 or 88 per cent of the samples agreed in the two tests; in 7 per cent of the cases, the precipitation test showed a more delicate reaction, being positive where the Wassermann was negative; in 5 per cent a negative precipitation reaction was obtained with a positive or a doubtful Wassermann.

It should be said here that of the 14 such instances, only 6 gave a definitely positive Wassermann (three or four plus reaction with both antigens), while 8 gave doubtful reactions (one or two plus reaction with cholesterinized antigen and negative with alcoholic antigen). About 2 per cent, therefore, of the tests gave a negative precipitation test with a definitely positive Wassermann. Of this 2 per cent (six tests), three of the patients had undergone vigorous treatment while histories of the other three were unobtainable. It is apparent, therefore, that vigorous treatment affects the result of the precipitation test under certain conditions.

Of the 22 instances in which a positive precipitation test was obtained with a negative Wassermann, all of these cases, so far as could be learned, gave a history of syphilis. The information was taken from the cards submitted to the laboratory with the blood samples and was not complete in all instances.

RELATION BETWEEN WASSERMANN AND PRECIPITATION TESTS

Whether this precipitation test is identical in the factors involved with the first part of the Wassermann test, has not been determined. Friedberger (8) claims that the amount of the actual precipitate is no criterion of the degree of the complement fixation. In fact the precipitating power of a serum may be destroyed by moderate heat without the destruction of complement-fixing antibodies. Dean (9) states that the proportion of antigen and antibody favorable for rapid and complete precipitation does not favor the most complete complement fixation. The two phenomena do not run parallel courses, but they probably represent different phases of the same phenomenon. Wells (10) sums up in this fashion:

> A favorite interpretation of the Wassermann reaction, which seems to harmonize with the known facts, is that there is a precipitation of serum globulin by the lipoidal colloids of the antigen, and adsorption of the complement by this precipitate.

An attempt was made by the present authors to separate the precipitate formed in the precipitation tests and to determine whether it was entirely responsible for the Wassermann reaction.

To this end, the precipitation tests were centrifuged at high speed for an hour, in order that as much as possible, if not all, of the precipitate might be thrown down. The supernatant fluid was then pipetted off and the sediment washed three times with salt solution. Wassermann tests were run on both, using of the supernatant fluid 0.2 cc. and all that was obtainable of the sediment, with two units of complement and thirty minutes incubation in the water bath.

The results are shown in table 2.

TABLE 2.

Result of Wassermann tests run on the supernatant fluid and the sediment of the precipitation tests

SPECIMEN NUMBER	WASSERMANN TEST ON SERUM	WASSERMANN TEST ON SUPERNATANT FLUID	WASSERMANN TEST ON SEDIMENT
1	4+	4+	4+
63	4+	4+	4+
2	4+	2+	4+
3	–	–	No precipitate
43	–	–	No precipitate
64	–	–	No precipitate

The precipitate in each case bound the complement, as was to be expected. In only one instance, however, was there a reduction in the strength of the Wassermann test run on the supernatant fluid. From these tests it is impossible to say whether the precipitate is entirely responsible for the binding of the complement or not. Further work needs to be done on the subject.

SUMMARY

1. A modification of the Sachs-Georgi precipitation test for syphilis is described, using clear blood serum and an alcoholic extract of beef heart, either cholesterinized or not.

2. The precipitation test agreed with the Wassermann test in 88 per cent of the cases; in 7 per cent it was more delicate, giving positive results where the Wassermann was negative; in 3 per cent it was negative where the Wassermann was doubtful; in 2

per cent it was negative where the Wassermann was positive. Treatment of the patient apparently affects the results of the precipitation causing it at times to come negative while the Wassermann still is positive.

3. Attempts to determine whether the precipitate formed in this test was entirely responsible for the Wassermann reaction were unsuccessful.

REFERENCES

(1) Klausner: Biochem. Zeitschr., 1912, **47**, 36.
(2) Lange: Berl. klin. Wochenschr., 1912, **69**, 897; Zeitsch. f. Chemiotherap., 1913, **1**, 44.
(3) Gammeltaft: Deutsche med. Wochenschr., 1912, **38**, 1934.
(4) Hirschfeld and Klinger: Deutsche med. Wochenschr., 1914, **40**, 1607.
(5) Vernes, A.: Presse Medicale, 1917, **25**, 704.
(6) Sachs and Georgi: Munch. med. Wochenschr., 1920, **67**, 66.
(7) Galli-Valerio: Corresp. Blatt f. Schweizer Aertze, 1919, **49**, 1977; Abstr. Jour. A. M. A., 1920, **74**, 563.
(8) Friedberger: Deutsche med. Wochenschr., 1906, **15**.
(9) Dean: Zeitsch. f. Immun., 1912, **13**, 84.
(10) Wells, H. G.: Chemical Pathology, 3d edition, p. 237.

A STUDY OF THE MECHANISM OF HUMAN ISOHEMAGGLUTINATION[1]

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The mechanism of isohemagglutination has been interpreted in different ways by the various investigators of this phenomenon. The assumption of differences of affinity as explanatory of the purely chemical nature of such antigen-antibody reactions, as Ehrlich hypothecated, has given way to the widely accepted principle that these bodies are of colloid nature and their interactions analogous to those of colloids in general. On the other hand, Gay (1) has offered the explanation that these reactions are governed by physicochemical laws; but this conception has not been supported by sufficiently convincing proof to warrant its acceptance.

Bordet (2) showed that bacterial agglutinins can be absorbed and specific agglutinins isolated by this method. The absorption of hemagglutinins was demonstrated by Malkoff (3), and later by Hektoen (4); since then, this has been observed repeatedly by others. Shibayama (5) and Ottenberg (6) showed that agglutinable cells will absorb more agglutinin than is necessary to agglutinate them, and the latter demonstrated this quantitatively by showing that one volume of agglutinable cells will absorb all the agglutinin from 16 volumes of agglutinative serum, whereas complete agglutination occurred with 13 volumes of serum. He also pointed out that when the ratio of serum to cells is 4 volumes to 6 volumes all the agglutinin is absorbed without producing agglutination, the explanation being that each of the cells absorbed some agglutinin but not enough to bring about clumping.

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By means of similar absorption experiments various investigators have attempted the classification of the bloods of humans and animals into groups according to their isoagglutination reactions. The efforts with human bloods have been successful, but no classifications of the bloods of other mammals have been definitely demonstrated. On the basis of these classifications assumptions have been made regarding the number of normal isohemagglutinins in human bloods and their specificity for particular agglutininogens or receptors of cells of particular groups. The specificity of normal human isohemagglutinins has been generally accepted, but has been denied by Landsteiner and Sturli (7) and others, and Karsner and Koeckert (8) recently pointed out that the specificity of these bodies in normal serum may be lost during the desiccation of such serums.

Isoagglutination of human erythrocytes was discovered independently by Landsteiner (9) and Shattock (10) in 1900. Landsteiner's classification provided for three groups, and he, followed by Descatello and Sturli (11), postulated two agglutinins and two agglutinophilic substances, or receptors, according to the following distribution:

Group I. Serum contains agglutinins A and B; cells possess no receptors.

Group II. Serum contains agglutinin A; cells possess receptor b.

Group III. Serum contains agglutinin B; cells possess receptor a.

Hektoen, in 1907 observed from the results of his absorption experiments, that

Cells I do not absorb agglutinin and therefore possess no receptors.

Cells II do not absorb the agglutinin of serum II for cells III, but do absorb the agglutinin of serum I for cells II and III.

Cells III do not absorb the agglutinin of serum III for cells II, but do absorb the agglutinin of serum I for cells II and III.

Cells II and III therefore possess distinct receptors and the failure of agglutination by some serums is due to the absence of suitable receptors.

He assumed the presence of three main agglutinins—the agglutinin in serum I for cells II and III, the agglutinin in serum II for cells III, and the agglutinin in serum III for cells II. By the independent discovery by Janský and Moss (12), a fourth group, whose serum contains no agglutinins but whose cells are agglutinated by all the other serums, was added. This phenomenon was also observed by Hektoen but not recognized by him as the basis of a separate group. Moss made the following classification, which has been widely adopted:

Group I. Serum is non-agglutinative; cells are agglutinated by serums II, III and IV.

Group II. Serum agglutinates cells I and III; cells are agglutinated by serums III and IV.

Group III. Serum agglutinates cells I and II; cells are agglutinated by serums II and IV.

Group IV. Serum agglutinates cells I, II, and III; cells are non-agglutinable.

In his explanation of these reactions, Moss assumed the presence of three different isoagglutinins and three isoagglutinophilic substances, as follows:

Group I. Serum contains no agglutinins; cells possess receptors a, b, and c.

Group II. Serum contains agglutinin A; cells possess receptors b and c.

Group III. Serum contains agglutinin B; cells possess receptors a and c.

Group IV. Serum contains agglutinins A, B and C; cells possess no receptors.

The contradiction between the hypotheses of Landsteiner and of Moss, as well as the questions raised by our experiments quoted above, determined the further investigation of the problem by the method of differential absorption. Throughout the remainder of this article the following classification, that of Janský, will be adhered to:

Group I. Serum agglutinates cells II, III and IV; cells are not agglutinable.

Group II. Serum agglutinates cells III and IV; cells are agglutinated by serums I and III.

Group III. Serum agglutinates cells II and IV; cells are agglutinated by serums I and II.

Group IV. Serum agglutinates no cells; cells are agglutinated by serums I, II and III.

Or graphically,

CORPUSCLES	SERUMS			
	I	II	III	IV
I	–	–	–	–
II	+	–	+	–
III	+	+	–	–
IV	+	+	+	–

Serums and thrice washed cells of the four groups were obtained, and the proof of their grouping established by testing with standard hemagglutinative serums of known groups. The agglutinative titer of the serums for suspensions of agglutinable cells of equal concentrations and the agglutinability of the washed cells by standard agglutinative serums were determined in order to permit accurate observations of the quantitative differences in the agglutinative power of the serums and the agglutinability of the cells used in the experiments. When cells IV were added to serum I, titer 1 to 32, in the ratio indicated by Ottenberg as proper for the absorption of all the agglutinin and complete agglutination of the cells, the mixture incubated at 37°C. for one hour, and centrifuged, it was seen that the serum had lost its agglutinative power for any cells and the cells would absorb no more agglutinin. When these agglutinated cells were washed with a quantity of saline equal to that of the serum used, the mixture heated at 50°C. for thirty minutes and centrifuged, the supernatant saline now possessed the power to agglutinate cells II, III and IV and behaved exactly as did the weakest potent dilution of the original serum I—that is, its agglutinative power was very slight; and the sedimented cells IV, after repeated washing with saline, reacted qualitatively with normal serums of the four groups exactly as normal cells IV, but quantitatively did not reabsorb all the agglutinin from an amount of serum I equal to that used in the original agglutination of these cells. Further, such cells after repeated washing did not absorb as much agglutinin from agglutinative serums of the other groups as was absorbed by an equal amount of normal cells IV. It was seen from this experiment that some, but not all of the absorbed agglutinins could be recovered from the agglutinated cells, and that the repeatedly washed cells reacted qualitatively exactly as did the normal cells. In other words, the original serum I was changed to a serum IV, the non-agglutinative saline reacted as a weak serum I, and the cells, repeatedly washed after agglutination, again became group IV cells, qualitatively.

By using cells II instead of cells IV with serum I, it was seen that the supernatant serum no longer agglutinated normal cells II, but did agglutinate cells III and IV; the agglutinated cells would absorb no more agglutinin from normal serums I or III; the agglutinated cells after heating at 50°C. gave up some of the absorbed agglutinin to the saline in which they were washed; the supernatant saline after centrifuging possessed the power to agglutinate cells II and IV, although only slightly; and the sedimented cells after repeated washing reacted qualtitatively as normal cells II, but failed to do so quantitatively. In this instance the original serum I may be said to have become a serum II; the supernatant saline reacted as a weak serum III; and the sedimented cells, after repeated washing, again reacted qualitatively as normal cells II. When this serum previously used to agglutinate cells II was added to cells III or IV, agglutination and complete absorption of the remaining agglutinins occurred; the agglutinated cells after heating gave up some of the absorbed agglutinin as evidenced by the subsequent agglutination of normal cells III or IV, by the supernatant saline and the reabsorption of agglutinin from normal agglutinative serums; and the sedimented cells after repeated washing recovered their original qualitative reaction to normal agglutinative serums. The results in this case were the same as those observed by treating normal serum II with normal cells III or IV; but by using a serum I previously treated with cells II, it is seen that, in addition to the above observations, serum I can be deprived successively of the agglutinins for cells II and III, or vice versa, or its entire agglutinative power may be lost by treatment with cells IV alone; and further, those agglutinins which agglutinate cells IV also agglutinate both cells II and III. In other words, serum I may be altered to react as serum II or III and then as serum IV, or as serum IV directly, according to the succession of the agglutinable cells used. Therefore, it is obvious that such fractional isolation of agglutinins from what may be called a polyvalent serum can be accomplished.

When cells II were treated with serum III in the same ratio as that in the above experiments, it was found that the serum had lost its agglutinins for any cells and the agglutinated cells would absorb no more agglutinin when treated with any agglutinative serum; so that, the combination of antigen and antibody was complete—no additional receptors remained uncombined, nor did the serum contain any additional agglutinins. However, when cells IV were treated with serum III, incubated, centrifuged, and a quantity of saline equal to that of the serum was added to the cells and heated, it was found that the cells, although agglutinated, absorbed other agglutinins from serums I or II; the serum III became non-agglutinative for any cells; the supernatant saline caused slight agglutination of normal cells II and IV; and the cells after repeated washing reacted qualitatively as normal IV cells. Before washing, however, the cells IV used in this instance reacted as normal cells III after treatment with serum III. When serum II was added to these agglutinated cells, the cells absorbed more agglutinin from that serum, and failed to absorb any additional agglutinin from serum I. These original cells IV were thus altered successively to react as cells III and then as cells I. Similar results were obtained by using serum II first, and then serum III; but when the cells were treated with serum I they did not absorb any further agglutinin from serums II or III. Therefore, it is also obvious that fractional saturation of receptors of what may be called polyvalent cells can be accomplished in a manner similar to the fractional absorption of agglutinins from polyvalent serums.

In order to prove the correctness or incorrectness of Moss' assumption, experiments were done, based upon this principle of fractional absorption of agglutinins and fractional saturation of receptors. Serum I was treated with cells II, incubated, centrifuged, and the serum recovered. According to Moss' hypothesis, this serum should be free from agglutinins B and C, and it was found that it no longer agglutinated cells II but did agglutinate cells III and IV. An equal volume of the same original serum I was treated with cells III, which, by the same reasoning, should then be free from agglutinins A and C. This serum was recovered after incubation and shown to have lost the agglutinin for cells III. The two recovered serums, one containing agglutinin A, and the other, agglutinin B, were then mixed and treated with cells IV. If these cells IV possessed receptors a, b, and c, the last would remain uncombined until further treated with a serum said to contain agglutinin C, namely, serum I. However, it was found after such treatment that no additional agglutinin had been absorbed from normal serum I which, after recovery, reacted *qualitatively* and *quantitatively* with equal amounts of the same suspensions of cells II, III and IV, used in the original determination of the titer of the serum, exactly as before. Therefore, cells IV do not possess a receptor c which remained uncombined, and serum I does not contain an agglutinin C which could have combined with such an agglutininogen.

The same results were obtained when cells IV were treated successively with serums II and III, and then with serum I. After treatment with serum II, the cells absorbed agglutinin from serums I and III only; but after subsequent treatment with serum III, the cells no longer absorbed agglutinin from serum I by virtue of an uncombined agglutininogen c of the cells and an agglutinin C in the serum. Similar experiments showed that cells II and III also do not possess more than one receptor.

That two agglutinins operate to produce the complete combination of receptors of cells of group IV can be shown quantitatively by the following experiment. The titer of serum I was determined for equal amounts of equally concentrated suspensions of cells II, III and IV and found to be 1:32, 1:32, and 1:64, respectively. When cells II were added to serum I in quantities sufficient to remove all the agglutinins for cells of that group, thus rendering that serum innocuous for the further agglutination of normal cells II, it was found that the titer of the recovered serum for cells IV was reduced to that of the original serum I for cells III, but the titer for cells III was unaltered. The same observation was made when cells III were used instead of cells II. This observation was also made in the preceding experiment (see page 535) in which one volume of serum I was treated with cells II and another volume with cells III, the serums recovered and mixed. The titer of each portion for cells IV was lower than that of the original serum I, but after combination of bothportions the titer of the mixture for cells IV was equal to that of the original serum I.

CONCLUSIONS

1. By what may be designated the fractional absorption of agglutinins from so-called polyvalent serums and the fractional saturation or combination of receptors of polyvalent cells, it can be shown that there are two distinct normal human isohemagglutinins and two agglutininogens, which operate to produce the group distribution of human bloods.

2. These agglutinins can be isolated by the method of fractional absorption.

3. Isohemagglutinins may be recovered from agglutinated corpuscles, but not completely.

4. By empirically designating the human isohemagglutinins A and B, and the agglutininogens or cell receptors a and b, their distribution may be conveniently charted as follows:

	GROUP			
	I	II	III	IV
Serum	AB	A	B	o
Cells	o	b	a	ab

5. The agglutinins in fresh normal serums are specific for particular agglutininogens or receptors.

REFERENCES

(1) **Gay**: Jour. Med. Res., 1908, 17, 321.
(2) **Bordet**: Ann. de l' Inst. Pasteur, 1901, 318.
(3) **Malkoff**: Deutsche Med. Wochenschr., 1901, 915.
(4) **Hektoen**: Jour. Inf. Dis., 1907, 4, 297.
(5) **Shibayama**: Centralbl. f. Bakt., 1902, 760.
(6) **Ottenberg**: Jour. Exp. Med., 1911, 13, 925.
(7) **Lansteiner & Sturli**: Wien. klin. Wochenschr., 1902.
(8) **Karsner & Koeckert**: Jour. Am. Med. Assoc., 1919, 73, 1207.
(9) **Landsteiner**: Zeitschr. f. Med., 1902, 3.
(10) **Shattock**: Jour. Path. and Bact., Feb., 1900.
(11) **Descatello & Sturli**: Münch. Med. Wochenschr., 1902, 49, 1090.
(12) **Moss**: Bull. Johns Hopkins Hosp., 1910, 21, 63.

IMMUNIZATION WITH BLACKLEG AGGRESSIN

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In the last three years the methods of immunizing calves against blackleg have been changed. The older methods of immunizing calves with small doses of virus, based on the work of Arloing, Thomas, and Wright have been largely replaced by sterile, soluble products derived from the blackleg bacillus. In 1917 Haslam and Franklin published a preliminary report (1) on the preparation and properties of natural blackleg aggressin. The publication did not have wide circulation, and several articles on the subject appearing subsequently (2) have failed to place the credit for the first practical use of a sterile blackleg immunizing agent in the United States. The work of Haslam and Franklin was the first demonstration of the practical value of sterile blackleg aggressin in actually controlling losses from blackleg on infected premises, although Roux (3) in 1888 and later Schobel (4) had shown by laboratory experiments that the sterile antigens possessed marked immunizing properties.

Roux collected the oedematous fluid from guinea-pigs dead after inoculation with blackleg, and filtered it through a Pasteur-Chamberland filter. He succeeded in immunizing other guinea-pigs against an otherwise lethal dose of blackleg virus. Roux suggested that the product might have value in the control of blackleg.

Schobel extended and confirmed Roux's conclusions, using calves in addition to guinea-pigs in his experiments. Schobel's technic of filtration and testing seems to have been at fault inasmuch as he stated, "The safest and quickest method (for testing sterility) is animal experiment, for it frequently occurs that the anaerobic culture process may prove the blackleg aggressin sterile, and at the same time guinea-pigs inoculated with 0.5 cc. of the aggressin may die of blackleg, in spite of the test." He showed doubt concerning the sterility of his product in a lengthy argument in which he proves that the immunizing value of blackleg aggressin cannot be due to the presence of a few organisms, but to specific soluble products.

MEDIA FOR TESTING STERILITY OF BLACKLEG AGGRESSIN

When our series of experiments on a sterile product for immunizing calves against blackleg was undertaken, we first concerned ourselves with the best method of ascertaining the sterility of blackleg aggressin. The use of ordinary culture media, such as glucose bouillon in fermentation tubes or glucose bouillon under oil, was speedily abandoned, because it required heavy inoculation of very viable material to produce growth of the blackleg bacillus. Following the work of von Hibler (5) and of Foth (6), we substituted the brain recommended by von Hibler for the meat pieces used in Foth's medium, and obtained a medium which seemed superior to either "Hirnbrei" of von Hibler, or "Meat piece liver bouillon" of Foth.

Grind liver, add one liter of water to each 500 grams of liver. Bring to a boil slowly and cook about 30 minutes or until the liquid is clear. Pour off the clear broth, add 1 per cent peptone and 0.5 per cent salt, adjust to a pH of 8, and it is ready to combine with the brain. Grind the brain, using the coarse grinder, and cook at 3 pounds pressure about one and one-half hours. Fill flasks three-fourths full with mixture of brain tissue and liver broth, and autoclave at 6 pounds for three hours.

This media will grow *B. chauveaui* and other anaerobes without protection from the air, and without preliminary boiling to drive off oxygen. Its reliability as a means of detecting minute traces of *B. chauveaui* in blackleg aggressin has been repeatedly checked, but one characteristic experiment will be cited. Dilutions of *B. chauveaui* culture were made in blackleg aggressin so that 20 cc. of the aggressin contained from one one-hundredth of a cubic centimeter to one one-billionth of a cubic centimeter of blackleg culture. For the guinea-pig tests the same culture and the same aggressin were used but the dilutions were prepared so that 10 cc. of aggressin contained from one-tenth to one five-thousandth of a cubic centimeter of the culture. The data are presented in table 1.

TABLE 1

Detection of B. chauveaui in aggressin by the use of brain medium and by the use of guinea-pigs

BRAIN FLASKS				GUINEA-PIGS			
Amount blackleg culture added	Number flasks	Time observed	Per cent in which blackleg detected	Amount blackleg culture added	Number guinea-pigs	Time observed	Per cent in which blackleg detected or guinea-pig died of blackleg
cc.		*hours*		*cc.*		*days*	
0.1				0.1	3	4	33⅓
0.05				0.05	3	4	66⅔
0.015	4	48	100	0.01	3	4	none
0.005	4	48	100	0.005	3	4	none
0.001	4	48	100	0.001	3	4	33⅓
0.0005	4	48	100	0.0005	3	4	66⅔
0.0001	4	48	100				
0.00005	4	48	100				
0.00001	4	48	100				
0.000001	4	48	100				
0.0000001	4	48	100				
0.00000001	4	48	100				
0.000000001	4	48	75				

From the data in table 1 we conclude that the brain medium is many times more delicate and more dependable than guinea-pigs as a test for the presence of *B chauveaui* in aggressin. A test on three guinea-pigs is not sufficient to demonstrate the absence of *B. chauveaui* in small quantities, but the test on brain medium is capable of detecting exceedingly minute amounts with a great degree of regularity.

METHOD OF PREPARING BLACKLEG AGGRESSIN

Blackleg aggressin is prepared from the oedematous fluid of calves dying in one to three days after inoculation with a pure culture of *B. chauveaui*. The methods of identifying the culture have been reported in a previous paper (7). The pure culture is prepared for calf inoculation simply by growing in brain medium, and straining through gauze. The oedematous fluid from calves dying of blackleg is collected partly by catching the fluid which comes out when the skin is removed and partly by pressing the affected tissue The oedematous fluid is cooled, centrifuged, and filtered through Berkfeld or Mandler filters.

After filtration blackleg aggressin is tested for sterility and for absence of toxicity. The sterility of blackleg aggressin is proven by adding a sample of at least 10 cc. to each of three brain flasks containing 250 cc. of medium, and incubating for at least sixty hours Absence of toxicity is established by inoculating guinea-pigs with 10 cc. of the aggressin, or calves with 50 to 100 cc. of the aggressin In these amounts blackleg aggressin rarely produces any local reaction, and very seldom any systemic reaction In our laboratories we have inoculated approximately 5000 guinea-pigs with 10 cc. each of sterile blackleg aggressin, and 150 calves with 100 to 50 cc. each of blackleg aggressin. In no instance have we demonstrated toxicity.

IMMUNIZING EXPERIMENTS ON GUINEA-PIGS

With this non-toxic, sterile product a number of immunization experiments have been carried out. On guinea-pigs the immunity, within limits, is proportional to the dose. This is illustrated by the experiment recorded in table 2, in which guinea-pigs were given varying amounts of blackleg aggressin, and later given one lethal dose of pure culture blackleg virus Guinea-pigs are immunized with difficulty with the smaller amounts of aggressin, although even 5 cc. of backleg aggressin gives marked protection. In 1917 we found that out of 1068 guinea-pigs inoculated with 10 cc. blackleg aggressin and later given 5 lethal doses of blackleg culture, 83.5 per cent lived. The amount of culture given was 0.5 cc. This proportion of immunity was later confirmed on about 4000 other guinea-pigs.

TABLE 2

Immunizing experiments on guinea-pigs

AGGRESSIN	NUMBER GUINEA-PIGS	VIRUS	NUMBER GUINEA-PIGS DIED	IMMUNE
cc.		*cc.*		*per cent*
0.5	10	0.1	8	20.00
2.0	12	0.1	8	33.33
3.0	11	0.1	5	55.60
5.0	33	0.1	11	66.66
10.0	32	0.1	6	81.25
Controls	18	0.1	17	5.55

IMMUNIZATION EXPERIMENTS ON CALVES

In calves as in guinea-pigs the result of an immunity test will depend on the amount of aggressin given, and the size of dose and the potency of the virus used in testing. Most of the tests on immunization of calves with blackleg aggressin have been made to determine some practical point in the preparation of the product and we have attempted to use the smallest amount of aggressin which would immunize against the largest amount of virus. After a number of experiments we found that 5 cc. of blackleg aggressin would usually protect against 5 cc. of pure culture virus. The virus used was of such strength that 0.1 cc. would kill two out of three guinea-pigs in forty-eight hours. In our preliminary report (1) a typical experiment was cited. We gave 8 cc. of aggressin to calves against 1 gram of dried meat virus which would kill guinea-pigs in 2 mgm. doses Under these conditions we got 100 per cent protection This method was satisfactory in general for demonstrating the protective action of the blackleg aggressin, but we did not feel it would show finer differences. That is a more sensitive test was required to determine such points as these; whether a certain preservative affected the potency of the product, or whether the oedematous fluid of a calf dying in seventy-two hours differed in value from the oedematous fluid of a calf dying in thirty hours. To obtain information on such points the dose of virus was increased so that occasionally a calf would succumb, and even the protected ones would show a moderate reaction. In an experiment to determine the effect of any factor on the potency of blackleg aggressin, a series of at least five calves is inoculated with aggressin prepared in the manner under consideration. Another series of the same size is inoculated at the same time with aggressin prepared in the usual manner. The two lots of aggressin should be of the same origin, or as nearly of the same origin as the conditions of the experiment will permit. The two series of calves should be inoculated with the same virus at the same time, along with at least five control calves. Although somewhat cumbersome and very expensive, the accuracy of the results seems to justify this method in ascertaining the fundamental principles involved in the production of blackleg aggressin.

TABLE 3

Immunization experiments on calves

DATE GIVEN AGGRESSIN	DATE GIVEN VIRUS	AGGRESSIN METHOD A		AGGRESSIN METHOD B		CONTROLS VIRUS ONLY	
		Died	Inoculated	Died	Inoculated	Died	Inoculated
May 29, 1919	June 18, 1919	1	4	0	5	5	10
June 6, 1919	June 30, 1919	2	4	1	4	4	5
June 21, 1919	July 8, 1919	0	6	0	5	4	5
July 1, 1919	July 16, 1919	1	5	0	5	5	5
July 5, 1919	July 22, 1919	2	4	0	5	3	5
Total....................		6	23	1	24	21	30

In table 3 is given a summary of five sets of experiments. Each set consisted of three groups of calves; one group which received no aggressin, and two groups vaccinated with 5 cc. of blackleg aggressin originally the same but subsequently treated in different manners. Two weeks after inoculation these two groups as well as the first were inocuated with 5 cc. of pure culture blackleg virus. It will be noted that 77 calves were used in the experiment. Of those receiving virus only, 21 out of 30 died in seventy-two hours. Of the 47 calves which received aggressin and virus only 7 died. Two of these, in the set inoculated June 6, 1919, one in each group, lived for over a week after receiving virus, and they must have had a considerable degree of immunity. The experiments given in the table satisfactorily demonstrate the protective action of blackleg aggressin, in addition to yielding certain information concerning the effect of different methods of treatment.

In the last three years natural blackleg aggressin has been used on five million calves, as nearly as can be estimated, and the field results have been highly satisfactory. The field results confirm the laboratory findings that in natural blackleg aggressin we have a sterile, non-toxic product, which confers active immunity against blackleg.

CONCLUSIONS

1. A medium is reported which is much more sensitive than guinea-pigs for detecting the presence of *B. chauveaui* in blackleg aggressin.
2. The immunity conferred on guinea-pigs by blackleg aggressin is proportional to the size of dose given. Ten cubic centimeters of blackleg aggressin will immunize about 80 per cent of guinea-pigs against five lethal doses of pure culture virus.
3. In calves as in guinea-pigs the result of an immunity test will depend upon the amount of aggressin given, and the size of dose and the potency of the virus used in testing. For detecting degrees of difference in aggressin, experiments were carried out in which the virus given was strong enough to cause a marked reaction in the protected calves. Under these conditions 74 per cent of calves receiving 5 cc. of blackleg aggressin were immune to 5 cc. of pure culture virus.
4. The findings set forth in the preliminary report concerning the immunizing value of blackleg aggressin have been confirmed and extended.
5. As closely as can be estimated, natural blackleg aggressin has been used on five million calves, and the field results have been highly satisfactory.

REFERENCES

(1) Haslam and Franklin: Circular No. 59, Agri. Exp. Sta., Kansas State Agri. College, January, 1917.

(2) Ward, H. C.: Jour. Am. Vet. Med. Ass., 1919, **55**, 394.

(3) Roux, L. E.: Ann. de l'Instit. Pasteur, 1888, **2**, 49.

(4) Schobel: Centralbl. f. Bakt. 1912, **62**, 296.

(5) Von Hibler: Handbuch der Pathogenen Microorganismen by Kolle and Wassermann, 1912, **4**, 800.

(6) Foth: Ztschr. f. Infectionskrankh. der Hausthiere, 1909. 239.

(7) Haslam and Lumb: Jour. Inf. Dis., 1919, **24**, 362–365.

A STUDY OF THE SPECIFICITY OF THE ABSORPTION OF ANTI-BACTERIAL PRECIPITINS

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In planning some experimental work on the immunological identity or non-identity of certain bacteria, it was found that a suitable agglutination antigen, that is, a stable suspension of uniform turbidity, could not be prepared from the cultures under consideration. This difficulty as is well known occurs with some bacterial types. The question therefore arose whether under the circumstances we could employ the precipitin reaction. Should sharp differences be noted with this reaction, non-identity would be established. Should, however, the antigens of two or more cultures be found to give equal or nearly equal precipitation with an immune serum it would be necessary to resort to the antibody absorption method to determine the ultimate identity or non-identity of such strains. How far would such absorptions reveal specific differences?

This question was raised because of the well known tendency of the precipitin-precipitinogen complex to bind immune bodies of all types as well as complement; a phenomenon utilized by Gay (1, 3), Chickering (1, 2) and by Weinstein (4) to separate the antibodies of immune sera. The question of the specificity of such an adsorption has apparently received little attention. Chickering found that an antigen of pneumococcus, type I or type II, removed only the immune substances for itself when a bivalent pneumococcus serum was employed. As these types are relatively very distinct and as they "cross-precipitate" to only a slight degree, these observations are of no significance as regards the question we have raised. Although we have not reviewed exhaustively the existing literature on bacterial precipitins, we do not believe that the fundamental question of the specificity of precipitin absorption has been studied with regard to bacteria which are closely related and therefore give similar or nearly identical reactions with precipitin sera.

We were in possession, fortunately, of cultures and serums which were especially suitable for a study of the above question. Smith and Ten Broeck (5) called attention to the extreme degree of cross agglutination between *B. typhosus* and the avian types *B. sanguinarium* and *B. pullorum* when an immune serum for any one of these varieties was employed.

Extending these observations to the precipitin reaction (6) we found that an anti-typhoid serum would precipitate the antigens of the above three types equally in the same dilutions and for the same time periods. The similarity of precipitation was so close that the strains could not be differentiated one from the other by this reaction (see following tables). We have also found that with an anti-pullorum serum similar cross reactions occur. Evidently, therefore, the serums and antigens of the three bacterial types mentioned above offer suitable reagents in testing the specificity of precipitin absorption. As a control, an antigen of *B. paratyphosus A* was used because antigens from this type were precipitated by the anti-typhoid serum in relatively low dilutions only. This type was therefore easily differentiated from *B. typhosus* by this reaction.

It was planned to carry out the absorptions in two ways; first, by utilizing the precipitin antigen; second, by utilizing the bacterial suspensions as is done in agglutinin absorptions.

The precipitin antigens[1] were prepared according to the method we (6) have previously reported. Briefly, this consists of growing the bacteria on large areas of agar; collecting and suspending them in distilled water; dissolving them by the addition of alkaline hypochlorite solution[2] and boiling; neutralization, precipitation by alcohol and the extraction of the sediment with

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[1] The term antigen is used in the sense of a reagent. The ability of antibody stimulation is not implied.

[2] For convenience we employed the commercially prepared solution, marketed as "Antiformin."

0.8 per cent salt solution at 100°C. The final extract is clarified by centrifuging. This method, as has been noted, yields a very concentrated antigen. The different antigens were made as comparable as possible by utilizing similar amounts of culture and extracting the end-products in the same volumes of saline. The anti-typhoid serum employed was from a horse (no. 659) which had received intravenous injections first of killed and then of live *B. typhosus* over a considerable period of time. The anti-pullorum serum was similarly prepared by utilizing rabbits.

The method employed in carrying out the absorptions with a precipitin antigen is illustrated by the following:

To 0.5 cc. (or other quantity as noted) of antiserum was added the absorbing antigen in the amounts stated in the tables. The mixtures were incubated for three hours at 45°C. with frequent shaking of the tubes, which were then placed in the refrigerator over night. The mixture was then centrifuged and the clear supernatant fluid used for the tests. The tests were carried out by mixing 0.1 cc. of the test antigen and 0.1 cc. of the absorbed serum in small tubes and incubating them at 45°C. for three hours, after which the results were read. The dilutions in the tables are the final dilutions of the original serum and include the dilution by the test antigen. Where the serum was absorbed, it includes the dilution by the absorbing antigen. The test antigen was the same as the absorbing antigen; both were used undiluted. The antigen was not diluted as this would reduce the precipitability of the antigen and therefore would reduce the range of action of the serum. Our previous work had shown that with the three closely allied types mentioned, dilution of the antigen gave no greater specificity

Tables 1 to 6 give the results obtained with the use of an anti-typhoid serum (horse no. 659) and an anti-pullorum serum (rabbit no. 339). Two different batches of antigens were employed to exclude the possibility of error due to irregularities in the strength of these reagents. As a further safeguard, similar experiments (not tabulated) were carried out with another anti-typhoid serum (horse no. 652). The results check those obtained with serum no. 659.

Tables 1 to 4 represent the results of two groups of absorption experiments. It will be noted that for each group of experiments

TABLE 1*

Precipitin tests—Anti-typhoid serum. Serum 0.5 cc. absorbed by 0.25 cc. of the precipitin antigens, and unabsorbed serum control

Absorbing antigens....	B. typhosus				B. sanguinarium				B. pullorum				B. paratyphosus A				Salt solution control			
Test antigens...	B. typhosus	B. sanguinarium	B. pullorum	B. paratyphosus A	B. typhosus	B. sanguinarium	B. pullorum	B. paratyphosus A	B. typhosus	B. sanguinarium	B. pullorum	B. paratyphosus A	B. typhosus	B. sanguinarium	B. pullorum	B. paratyphosus A	B. typhosus	B. sanguinarium	B. pullorum	B. paratyphosus A
Serum dilutions:																				
1:3	1	1	—	—	1	1	—	—	—	—	—	—	++	++	++	—	++	++	++	+1
1:6	—	—	—	—	—	—	—	—	—	—	—	—	++	++	++	—	++	++	++	+
1:12	—	—	—	—	—	—	—	—	—	—	—	—	+1	+1	+1	—	+1	+1	+1	±
1:24	—	—	—	—	—	—	—	—	—	—	—	—	+	+	+	—	+	+	+	1
1:48	—	—	—	—	—	—	—	—	—	—	—	—	1	1	1	—	±	±	±	—
1:96	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1	1	1	—

* This table is representative of the results obtained with larger absorbing doses of antigen. A duplicate experiment using also 0.25 cc. of antigen showed no reaction in a 1:3 dilution with the first three types.

Symbols: ++, +1, +, ±, 1, degrees of reaction from strongest to weakest. Degree of reaction obtained with the 1:3 serum dilution with its homologous antigen taken as ++ for comparative readings.

TABLE 2

Precipitin tests—Anti-typhoid serum. Serum 0.5 cc. absorbed by 0.1 cc. of the precipitin antigens, and unabsorbed serum control

Absorbing antigens	**B. typhosus**			**B. sanguinarium**			**B. pullorum**			**Salt solution control**		
Test antigens...............	**B. typhosus**	**B. sanguinarium**	**B. pullorum**	**B. typhosus**	**B. sanguinarium**	**B. pullorum**	**B. typhosus**	**B. sanguinarium**	**B. pullorum**	**B. typhosus**	**B. sanguinarium**	**B. pullorum**
Serum dilutions:												
1:3	±	+	±	±	±	—	±	±	1	++	++	++
1:6	1	±	1	1	1	—	1	±	—	++	++	++
1:12	1	1	—	—	—	—	1	1	—	++	++	++
1:24	—	—	—	—	—	—	—	—	—	+	+1	+
1:48	—	—	—	—	—	—	—	—	—	±	±	±

TABLE 3

Precipitin tests—Antityphoid serum. Serum 0.5 cc. absorbed by bacterial mass, and unabsorbed serum control

Absorbing bacterium...	B. typhosus				B. sanguinarium				B. pullorum				B. paratyphosus A				Salt solution control			
Absorbing dose........	9 agar slants Mass=0.2 cc.*				6 agar slants Mass=0.08 cc.				9 agar slants Mass=0.15 cc.				6 agar slants Mass=0.08 cc.				—			
Test antigens...	B. typhosus	B. sanguinarium	B. pullorum	B. paratyphosus A	B. typhosus	B. sanguinarium	B. pullorum	B. paratyphosus A	B. typhosus	B. sanguinarium	B. pullorum	B. paratyphosus A	B. typhosus	B. sanguinarium	B. pullorum	B. paratyphosus A	B. typhosus	B. sanguinarium	B. pullorum	B. paratyphosus A
Serum dilutions:																				
1:3	–	–	–	–	–	–	–	–	–	–	–	–	++	++	++	–	++	++	++	+1
1:6	–	–	–	–	–	–	–	–	–	–	–	–	++	++	++	–	++	++	++	+
1:12	–	–	–	–	–	–	–	–	–	–	–	–	++	++	++	–	++	++	++	±
1:24	–	–	–	–	–	–	–	–	–	–	–	–	+	+	+	–	+1	+1	+	1
1:48	–	–	–	–	–	–	–	–	–	–	–	–	±	±	±	–	±	±	±	–
1:96	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1	1	1	–

* Approximate mass of bacilli after sedimenting suspension of growth from agar slants. Larger absorbing masses gave the same results.

TABLE 4

Precipitin tests—Anti-typhoid serum. Serum 0.5 cc. absorbed by bacterial mass, and unabsorbed serum control

Absorbing bacterium........	B. typhosus			B. sanguinarium			B. pullorum			Salt solution control		
Absorbing dose.............	6 agar slants Mass=0.08 cc.			3 agar slants Mass=0.04 cc.			6 agar slants Mass=0.08 cc.			—		
Test antigens...............	B. typhosus	B. sanguinarium	B. pullorum	B. typhosus	B. sanguinarium	B. pullorum	B. typhosus	B. sanguinarium	B. pullorum	B. typhosus	B. sanguinarium	B. pullorum
Serum dilutions:												
1:3	+	+	+	±	±	+	+	+	+	++	++	++
1:6	1	1	1	–	–	–	±	+	±	++	++	++
1:12	–	–	–	–	–	–	1	±	1	++	++	++
1:24	–	–	–	–	–	–	–	1	–	+1	+1	+
1:48	–	–	–	–	–	–	–	–	–	±	±	±
1:96	–	–	–	–	–	–	–	–	–	1	1	1

TABLE 5

Precipitin tests—Anti-pullorum serum. Serum 0.6 cc. absorbed by 0.1 cc. of the precipitin antigens, and unabsorbed serum control

ABSORBING ANTIGENS	B. typhosus			B. sanguinarium			B. pullorum			Salt solution control		
TEST ANTIGENS	B. typhosus	B. sanguinarium	B. pullorum	B. typhosus	B. sanguinarium	B. pullorum	B. typhosus	B. sanguinarium	B. pullorum	B. typhosus	B. sanguinarium	B. pullorum
Serum dilutions:												
1:3	–	–	–	–	–	1	1	–	1	++	+1	++
1:6	–	–	–	–	–	–	–	–	–	+1	+	+1
1:12	–	–	–	–	–	–	–	–	–	1	–	–
1:24	–	–	–	–	–	–	–	–	–	–	–	–

TABLE 6

Precipitin tests—Anti-pullorum serum. Serum 0.6 cc. absorbed by bacterial mass, and unabsorbed serum control

ABSORBING BACTERIUM	B. typhosus			B. sanguinarium			B. pullorum			Salt solution control		
ABSORBING DOSE	3 agar slants Mass = 0.1 cc.			3 agar slants Mass = 0.1 cc.			3 agar slants Mass = 0.1 cc.			–		
TEST ANTIGENS	B. typhosus	B. sanguinarium	B. pullorum	B. typhosus	B. sanguinarium	B. pullorum	B. typhosus	B. sanguinarium	B. pullorum	B. typhosus	B. sanguinarium	B. pullorum
Serum dilutions:												
1:3	–	–	–	1	–	1	–	–	–	++	+1	++
1:6	–	–	–	–	–	–	–	–	–	+1	+	+1
1:12	–	–	–	–	–	–	–	–	–	1	–	–
1:24	–	–	–	–	–	–	–	–	–	–	–	–

one table gives the smallest absorbing dose which resulted in complete or practically complete absorption and the succeeding table gives the next smaller absorbing dose which failed to absorb completely. The experiments are presented in this way to show that we have carefully considered the size of the absorbing dose in relation to the specificity or non-specificity of the results. It may seem that we should have used further intermediate doses. But even with the doses recorded, repeated experiments showed some variation in the degree of absorption; the limits of the delicacy of the method were, therefore, evidently reached. Furthermore, with the dose which did not wholly absorb, it is evident that the reduction is approximately the same with each of the antigens. In one instance (table 2), there is a greater reduction for one antigen (pullorum), which, at first glance, seems to indicate a specific difference. As this reduction is almost equally marked in the case of absorption by antigens of the other two types, this difference must be referable to the test antigen. This brings up the question of our inability to standardize adequately precipitin antigens, which may be a factor in the non-specificity shown with absorption by antigens which precipitate equally. This question is considered more in detail below.

It is evident from the tables 1 to 6 that the absorption of precipitins from the immune serums by the use of precipitin antigens, or even by whole bacteria, was specific only within narrow limits. With a heterologous type which gave little precipitation, the absorption by the antigen of this type removed only the precipitins against itself (see *B. paratyphosus A*, tables 1 and 3). In the case of the heterologous types giving profuse precipitates, absorption by one of these types resulted in the complete removal of all the precipitins, even those active against the type homologous to the serum.

Assume in the case of the above cultures, that satisfactory agglutinating antigens were impossible of preparation, and reliance had to be placed upon the precipitin reaction. If we allow this assumption, it is obvious that the precipitin absorption would have given unsatisfactory results where its application would be required because of failure of differentiation due to cross-precipitation and would have given satisfactory results where it was not required because differentiation was evident on direct precipitation.

The results thus far recorded as regards non-specificity of absorption by the precipitin antigen itself were more or less expected. The result with absorption of precipitins by the bacterial bodies (tables 3 and 4) was so unexpected that it raised the question as to how much these sera cross-agglutinated and consequently what influence an equal absorbing mass of bacteria would have on the agglutinin content. The degree of

TABLE 7

Agglutination tests—Anti-typhoid serum. Unabsorbed serum

SERUM DILUTIONS	HORSE 659			HORSE 532		
	B. typhosus	B. sanguinarium	B. pullorum	B. typhosus	B. sanguinarium	B. pullorum
1:150	++	++	++	++	++	++
1:1500	++	±	+	++	++	++
1:15,000	++	1	1	++	1	1
1:150,000	++	–	–	+	–	–
1:300,000	+1	–	–	±	–	–
1:600,000	+1	–	–	–	–	–
1:1,200,000	+	–	–	–	–	–
Control	–	–	–	–	–	–

Symbol ++ = complete reaction, other symbols indicate decreasing degrees of reaction.

Method.—Broth antigens (Dreyer) employed. Tests incubated for three hours at 45°C. Readings made after sedimentation in ice-chest over night.

TABLE 8

Agglutination tests—Anti-pullorum serum. Unabsorbed serum

SERUM DILUTIONS	B. TYPHOSUS	B. SANGUINARIUM	B. PULLORUM
1:200	++	++	++
1:400	+1	++	++
1:800	+	+1	+1
1:1600	±	±	+
1:3200	1	1	±
1:6400	–	–	±
Control	–	–	–

cross-reaction as regards agglutination is given in tables 7 and 8, and is of interest in showing how the cross-reaction by these two antibodies may or may not run parallel. In the case of the anti-typhoid serum the cross-agglutination is very much less marked than that recorded by Smith and Ten Broeck.

When the effect of a similar mass of bacteria (as above) on the agglutinin content of the serums was investigated, it was found that absorption of the serums by the homologous bacteria, using the same masses as in connection with the precipitin tests described above, reduced the specific titer only to a slight degree. Absorption by a heterologous strain, in the doses noted, also had relatively little influence on the height of the specific or group agglutinations. However, if the group titer was low, the heterologous strain might serve to remove the agglutinins for the absorbing type, but would leave the specific titer nearly unchanged.

The non-specific results obtained with precipitin absorption in the above experiments induced us to try other related varieties of bacteria and their antisera in the attempt to verify the astonishing results recorded above.

Antigens were prepared from three strains; a typical *B. cholerae suis* no. 131, an atypical suis variety no. 333 and a *B. paratyphosus B*. These were tested with an anti-serum for *B. cholerae suis*. The results are not tabulated. Only a moderate direct cross precipitin reaction was obtained. Precipitin absorption of this serum was also carried out by using bacterial masses of each variety for absorbing. The absorption was incomplete in some instances. Where it was complete, specificity was shown and where absorption was incomplete, the indication of specificity was so marked that further experiments with this serum were not made. These results indicate again that with slight or moderate cross-precipitation, specific results can be obtained by absorption.

The atypical strain of porcine origin mentioned above was then tested with *B. paratyphosus B* serum and, as expected on the basis of our previous agglutinative study of these strains, rather marked cross-precipitation was obtained. Absorptions of this serum was carried out with the atypical *B. cholerae suis* type and with *B. paratyphosus B*, using bacterial masses for absorption.

The precipitin experiments with this serum and these two bacterial types are recorded in table 9. All the tests made are given for comparison. These experiments are of especial interest because they record the greatest fluctuation in the degree of cross-reaction that we have encountered. This variation in the direct reaction seems to parallel the degree of specificity obtained after absorption. Thus, in the "three slant" series the cross-reaction is least marked in the salt solution control and likewise the specificity after absorption is noticeable. In the "six slant" series where the degree of cross-reaction in the salt solution control is more marked, the specificity after absorption is so slight as to be neglible. In the "nine slant" series, although the cross-reaction is again less, the absorption is complete and non-specific.

The degree of cross-agglutination as well as the results of agglutinin absorption with the above two strains are given in table 10.

Two questions have probably occurred to the reader in a consideration of the above protocols. First, why resort to absorption when a difference in direct precipitation is encountered comparable to that shown by the two strains given in table 9.

TABLE 9

Precipitin tests—Anti-paratyphosus B serum. Serum 1 cc. absorbed with bacterial mass, and unabsorbed serum control

ABSORBING TYPE........	B. paratyphosus B						B. suis atypical No: 333						Salt solution control					
ABSORBING DOSE......	3 slants Mass=0.1 cc.		6 slants Mass=0.2 cc.		9 slants Mass=0.28 cc.		3 slants Mass=0.1 cc.		6 slants Mass=0.25 cc.		9 slants Mass=0.29 cc.		Of 3 slant series		Of 6 slant series		Of 9 slant series	
TEST ANTIGENS........	B. paratyphosus B	B. suis 333	B. paratyphosus B	B. suis 333	B. paratyphosus B	B. suis 333	B. paratyphosus B	B. suis 333	B. paratyphosus B	B. suis 333	B. paratyphosus B	B. suis 333	B. paratyphosus B	B. suis 333	B. paratyphosus B	B. suis 333	B. paratyphosus B	B. suis 333
Serum dilutions:																		
1:3	+	1	—	—	—	—	+1	1	±	—	—	—	++	+	++	+1	++	+1
1:6	±	—	—	—	—	—	+1	—	—	—	—	—	++	+	++	+1	++	+
1:12	1	—	—	—	—	—	±	—	—	—	—	-	+1	±	+1	+	+1	±
1:24	—	—	—	—	—	—	—	—	—	—	—	—	±	1	+	1	±	1
1:48	—	—	—	—	—	—	—	—	—	—	—	—	1	—	1	—	—	-

TABLE 10

Agglutination tests—Anti-paratyphosus B serum. Serum absorbed by bacterial mass, and unabsorbed serum control*

ABSORBING TYPE	B. paratyphosus B*		B. suis. atypical No. 333*		Salt solution control	
TEST ANTIGENS	B. paratyphosus B	333	B. paratyphosus B	333	B. paratyphosus B	333
Serum dilutions:						
1:50	–	–	+1	–	++	+1
1:100	–	–	++	–	++	++
1:200	–	–	++	–	++	++
1:400	–	–	++	–	++	++
1:800	–	–	++	–	++	++
1:1600	–	–	++	–	++	++
1:3200	–	–	++	–	++	++
1:6400	–	–	+1	–	++	+1
1:12,800	–	–	+1	–	+1	+
1:25,600	–	–	±	–	+	±
1:51,200	–	–	–	–	1	–
Control	–	–	–	–	–	

* To one volume of packed bacteria was added three volumes of serum diluted 1:15, the mixture incubated at 37°C., shaking frequently, ice chest overnight and centrifuged. The clear supernatant fluid used for further dilutions.

TABLE 11

Precipitin tests—Antiparatyphosus B serum. Effect of dilution of precipitin antigens on the degree of cross or group reactions

ANTIGENS. Kind	"Antiformin"						Broth	
Of	B. paratyphosus B			B. suis 333			B. paratyphosus B	333
Dilution	0	1;10	1:50	0	1:10	1:50	0	0
Serum dilutions:								
1:3	++	±	1	+1	±	1	±	1
1:6	++	±	1	+1	±	–	1	1
1:12	+1	1	–	+	1	–	1	–
1:24	+	1	–	±	–	–	1	–
1:48	1	–	–	–	–	–	–	–

In answer to this we would say that the degree of difference elicited in these tests (before absorption), even where most marked, is not appreciably greater than that we have previously encountered with two bacteria which were identical, except as regards their sensitiveness to antibody action. Furthermore, as these differences might be accentuated by inequalities in the antigens, it is evident that the direct reaction could not be accepted as conclusive unless it showed a more marked differentiation.

The second question would be in relation to the dosages recorded. Would the moderate differences in the absorption dose result in a loss of specificity with agglutinin absorption similar to the apparent loss of specificity with the precipitin absorptions recorded in table 9?

On the basis of a very extensive experience with the agglutinin absorption method, we feel justified in answering in the negative. For instance, if a dose of 0.1 cc. of a heterologous bacterium absorbed the group agglutinins for itself and because of very close relationship with the serum strain reduced the titer somewhat for the latter, double or triple this absorption dose would only result in some further reduction, not necessarily very marked. In no instance, thus far, even with excessively large doses, have we observed a complete removal of the specific agglutinin from a highly potent serum by the use of a heterologous strain.[3]

In other words, the agglutinin-absorption technic leaves us a practical and relatively wide working range as regards dosage. The precipitin absorption method on the contrary, as the experiments indicate, has a narrow range and may fail to reveal distinct differences, especially where strains are closely related, even with scrupulous regard as to the size of the absorption dose.

We have already referred to our previous failure to obtain more specific precipitation results by dilution of the antigens of *B. typhosus*, *B. sanguinarium* and *B. pullorum*. As these results might not hold true with other closely related bacteria, it seemed desirable, as a further control, to determine this point for other types. The supernatant fluid of centrifuged broth cultures was also used as an added control antigen on the specificity of the heated antigens.

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[3] In this connection it is worthy of note that some workers have utilized a method for agglutinin absorption which does not include testing below 50 per cent or 25 per cent of the original titer of the serum. This inadequate method has even been employed to separate "antigenic varieties" of a definite type of bacterium, such as *B. typhosus*, for instance. Such reports have been given a prominence which critical scrutiny does not warrant.

Table 11 gives the results of this experiment. There is little suggestion of greater specificity in the dilute antigens, with the exception, possibly, of the clarified broth antigen which in itself is a dilute antigen. Even if we exclude the probable influence of differences in concentration in the case of the broth antigens, it is evident that these antigens would be of little value in carrying out the absorption technic because the initial differences before absorption are very slight, and founded on the readings of the minimal observable reaction. A very small absorbing dose would suffice to reduce this reaction beyond the limit of certainty of observability. In other words, dilution reduces the degree of reaction obtained with the antigens and leaves us no working range for absorption.

The results of precipitin tests made after absorption of antimeningococcus serums type I and type II (Gordon) are given in tables 12, 13 and 14.

The results with type I serum are sharply specific with the absorbing dose employed. The results with type II serum are

TABLE 12

Precipitin tests—Antimeningococcus serum, type I. Serum 1 cc. absorbed by bacterial mass, and unabsorbed serum control*

ABSORBING TYPE	I			II			III			Salt solution control		
ABSORBING DOSE	15 billion†			15 billion			15 billion			—		
TEST ANTIGENS	I	II	III	I	II	III	I	II	III	I	II	III
Serum dilutions:												
1:3	1	−	−	+1	−	−	+1	−	−	++	++	+
1:6	−	−	−	+1	−	−	+1	−	−	++	++	±
1:12	−	−	−	+	−	−	+	−	−	+1	±	1
1:24	−	−	−	±	−	−	1	−	−	+	−	−
1:48	−	−	−	−	−	−	−	−	−	1	−	−

* Similarly specific results were obtained with another set of antigens in spite of the fact that the type I antigen was decidedly weaker than those of the other types.

† Estimated by opacity standards.

TABLE 13

Precipitin tests—Antimeningococcus serum, type II. Serum 1 cc. absorbed by bacterial mass and unabsorbed serum control

ABSORBING TYPE	I			II			III			Salt solution control		
ABSORBING DOSE	15 billion			15 billion			15 billion			—		
TEST ANTIGENS*	I	II	III	I	II	III	I	II	III	I	II	III
Serum dilutions:												
1:3	−	±	−	±	±	±	1	±	−	+	+1	+
1:6	−	±	−	1	±	1	−	±	−	1	+	1
1:12	−	1	−	−	1	−	−	1	−	−	±	−
1:24	−	1	−	−	−	−	−	1	−	−	1	−
1:48	−	−	−	−	−	−	−	−	−	−	−	−

* Antigens, Lot no. 1, of which type I is distinctly weaker than the other two.

TABLE 14

Precipitin tests—Antimeningococcus serum, type II. Serum 1 cc. absorbed by bacterial mass and unabsorbed serum control

ABSORBING TYPE	I			II			III			Salt solution control		
ABSORBING DOSE	20 billion			20 billion			20 billion			—		
TEST ANTIGENS*	I	II	III	I	II	III	I	II	III	I	II	III
Serum dilutions:												
1:3	1	1	−	1	1	−	+1	+1	−	++	++	±
1:6	−	1	−	−	1	−	±	+	−	++	+1	1
1:12	−	−	−	−	−	−	1	1	−	+	±	−
1:24	−	−	−	−	−	−	−	−	−	1	1	−
1:48	−	−	−	−	−	−	−	−	−	−	−	−

Antigens, Lot 2, of which type II seems somewhat the weaker.

irregular and to some extent contradictory. In one case, table 13, we knew that the type I antigen was less strong than the other but we used it to see what results would develop. Here there is an apparent specificity, but obviously this specificity may be wholly illusory and due to inequalities in the antigen. It will be noted that the absorption by the homologous strain is incomplete. This test could not be repeated to complete exhaustion as the supply of these antigens was depleted.

A repetition of the experiment with another set of antigens, table 14, led to results so nearly, if not quite non-specific, that further tests were not made.

The finished antigen of type II in this series seemed somewhat less concentrated than the others. This may be a factor in the apparently non-specific results. This again brings forward the question as to the influence of the antigen on the results obtained. The results already recorded depend on the use of similar masses of culture and their manipulation in a similar way, the end antigens being assumed therefore to be closely comparable. In other words, we have at present no practical method by which we can determine the concentration of precipitable substance in an antigen and its consequent power to react. One cannot exclude the possibility that if we had such a criterion, more nearly specific results might be obtained. We wish to make clear, therefore, that the non-specific results presented are limited to our present available methods. Considering our results as a whole, we are inclined to believe that even with a satisfactory method for antigen standardization, non-specific results would still occur where cross-precipitation was marked.

The results obtained parallel the non-specific results occasionally noted with a low titer agglutinating serum, which phenomenon may be explained by the assumption that the increased agglutination titer is due to an accumulation of group and normal agglutinins, with only a negligible associated increase in specific agglutinins. It would seem, therefore, that the non-specific results obtained with the precipitin absorptions were likewise due to a low titer of the serum. The sera employed were not of a low titer in the usually accepted sense. They were from animals receiving intensive immunization and compared favorably with the most active precipitating serums we have been able to produce in the past. We should, therefore, prefer to consider the range of action as narrow, not low. The narrowness of the working range may have influenced the results, but it should be noted that the methods employed included a possibility of reduction from 48 to 3 or one-sixteenth of the reacting titer. A similar ratio of possible agglutinin reduction with an agglutinating serum would yield specific results if the same care were employed as regards the absorbing dosage.

In this connection, and also to illustrate the statements already made, we have given the agglutinin absorption results with *B.*

paratyphosus B serum in table 10. This table shows the clear-cut results obtainable even when the cross-agglutination approximates 100 per cent. These results are not selected and show the clean cut results obtainable even when the absorbing dose is estimated and not determined by titration.

CONCLUSIONS

A precipitin absorption method has only a limited application in the differentiation of closely related types of bacteria which, because of such relationship, show marked cross-precipitation. The tendency in such a case is toward non-specific results; that is, the bacterium, heterologous to the serum, in removing the precipitins active against itself may also remove the precipitins active against the homologous type. Where the cross-precipitation is less marked, more specific results tend to appear after precipitin absorption.

The results are the same whether a precipitin antigen or the bacteria themselves are used for absorption.

The results may be influenced by our lack of a suitable method for the standardization of precipitin antigens of bacterial origin. The conclusions as given, therefore, are stated in terms of this limitation.

The non-specific results obtained may be referable, in some degree, to the narrow working range of precipitating sera.

The inadequacy of the precipitin reaction as a primary or single method for the differentiation of related bacteria, which are serologically still unclassified, is again emphasized. While similar failures in differentiation may occur with the agglutination reaction, such failures may be easily corrected by resorting to the absorption method.

REFERENCES

(1) Gay, F. P., and Chickering, H. P.: Journ. Exp. Med. 1915, **21**, 389.
(2) Chickering, H. P.: Journ. Exp. Med. 1915, **22**, 248.
(4) Weinstein, I.: Journ. Immunol., 1918, **3**, 17.
(3) Gay, F. P., and Stone, R. L.: Journ. Immunol., 1916, **1**, 83.
(5) Smith, T., and Ten Broeck, C.: Journ. Med. Res., 1915, **31**, 503 and 547.
(6) Krumwiede, C., and Noble, W. C.: Journ. Immunol., 1918, **3**, 1.

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